Nest Defence Behaviour of *Vespula vulgaris*:

Ecological and Molecular Approaches

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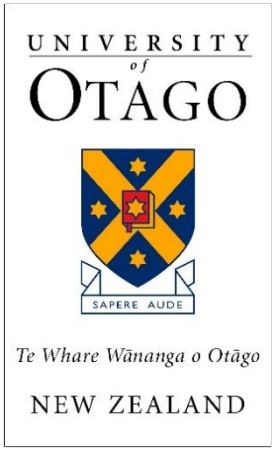

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For the brave epidemiologists, pathologists, virologists, and many other scientists who dedicate their lives to save those of others.

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Abstract

In behavioural ecology, aggression is comprised of a suite of agonistic behaviours displayed by animals during confrontations, which may or not involve direct fights between groups or individuals. Aggression serves a range of ecological functions, including competing for resources, subjugating prey, self-preservation, and brood defence. Aggressive behaviours are near ubiquitous across animal taxa and have been theorized to play a major role in biological evolution.

Social Hymenoptera (ants, social bees, and social wasps) are renowned for their expressive aggression behaviours, especially in the context of nest defence. Being able to defend their brood against predators is essential to ensure colony survival and reproduction, and aggression has been suggested as a main trait underlying the ecological success of social insects and the evolution of eusociality. Many mechanisms have been suggested to help drive defensive aggression in the social Hymenoptera, including abiotic, social, and molecular factors.

In this thesis, I studied the nest defence behaviour of *Vespula vulgaris*. This social wasp, an introduced pest species in New Zealand, is an ideal model to study mechanisms underlying aggression behaviours due to its dense populations, quantifiable response to simulated predator attacks, and variation in aggression phenotype.

I start with a systemic review of worldwide predation pressures on social wasp colonies. I describe the taxonomic diversity of predators of wasp individuals and colonies, and how they vary across different social wasp taxa in different areas of the world. Based on my findings, I draw conclusions on how predator-prey interactions are shaped by their behavioural ecology, and make inferences on how these relationships might have developed over the groups' evolutionary history.

I then investigated how *V. vulgaris* nest defence varies according to age and experience over time at the colony level in a field setting. I found that colony aggression fluctuates over the colony cycle, but provide no evidence towards the effect of habituation or sensitization over time as colonies experience continuous simulated predator attacks. I relate these findings to shifts in the colonies' physiological age, interpreted in the context of natural

demographic changes happening over the course of the colony's seasonal cycle. I also provide insights on the absence of abiotic effects on colony-level aggression.

Finally, I investigated individual brain transcriptome differences between nest defenders and foragers, aiming to identify genomic bases for aggressive behaviour variation within and across colonies. I found that while colonies have differing aggressive phenotypes and distinct transcriptomic patterns, very few genes are differentially expressed between behavioural castes within colonies. I briefly discuss the biological significance of these genes of interest, and provide ideas for further investigating the study of aggression at the molecular level.

Overall, this thesis contributes to the advancement of the field of behavioural ecology by providing evidence on different mechanisms that may play different roles at determining consistent behavioural variation in a model study system. Although there are many challenges and limitations for field-based studies, such as sub-optimally standardized experiments and snapshot phenotypical sampling, by investigating ecological and molecular influences on aggression behaviours in *V. vulgaris* I add to the current knowledge on how behavioural variation has shaped the evolution of eusociality in Hymenoptera.

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Chapter One

General Introduction

1.1 AGGRESSION BEHAVIOUR IN ANIMALS

1.1.1 Defining Biological Aggression

Aggression as a biological phenomenon

Agonistic interactions between organisms, including aggression displays and fights, are one of the main forces underlying animal evolution (Darwin, 1859, 1871; Lorenz, 1966; Archer 1988; Rittschof and Grozinger 2021). Aggressive individuals can be highly successful in capturing prey, excluding competitors, or eliminating threats, which leads to greater fitness values (Sih, Bell et al. 2004a, Peiman and Robinson 2010). Even the most basic of organisms exhibit forms of agonistic behaviour (King 2004); for instance, some protozoans can use chemical cues to elicit anti-predator responses (Kusch 1999). In animals, aggressive contests are observable throughout a diverse range of phyla (Peiman & Robinson, 2010), from the more spectacular displays such as mating competition fights in deer (Clutton-Brock, Albon et al., 1979) and cannibalization during feeding frenzies in sharks (Clua, Chauvet et al., 2013), to the physical removal and exclusion of neighbouring competitors in sessile cnidarians (Chadwick, 1987) and limpets (Stimson, 1970). Aggression, in its many forms, seems to be near ubiquitous across animal taxa.

A brief history of the research of animal aggression

The definition of aggression in a biological context has historically been the subject of some dispute among behavioural ecologists. In his book "On Aggression", ethologist Konrad Lorenz defines biological "aggression" as one of the many "fight-like behaviours" displayed by animals (Lorenz, 1966). Lorenz's perspective on aggression is restricted to what is currently referred to as intraspecific or competitive aggression (Archer, 1988); in other

words, an aggressive animal is one who tends to initiate fights with its conspecifics.

More recently, the meaning of "aggression" is often used to encompass a wider range of behaviours. John Archer, in "The Behavioural Biology of Aggression", made the first comprehensive attempt to review the literature on aggression under the light of behavioural ecology and sociobiology (Archer, 1988). Archer's understanding of aggression includes both intra and interspecific agonistic displays, which may occur under varying ecological contexts (e.g. protective aggression, parental aggression, competitive aggression; Archer 1988).

Defining aggression

Here, I partially borrow from previous perspectives and define aggression in animals as a functional group of behaviours involving direct confrontation between two or more animals, within or across species (Scott and Fredericson 1951; Archer, 1988). When discussing aggression, I refer to behaviours that may be observed in actual fights between animals, but also include confrontational displays. Considering domestic dogs as an example, the acts of biting, growling, or bearing of teeth at another animal can all be included as "aggressive" behaviours (Crowell-Davis, 2008), even if the latter two do not include direct contact between individuals.

1.1.2 The Role of Aggression in Animal Ecology and Evolution

Defensive aggression

Aggression may occur in diverse biological contexts and can be adaptive to varying degrees (Sih et al. 2004a). From a proximate perspective, defensive behaviours are perhaps the most intuitive examples of the adaptiveness of aggression. The execution of aggressive behaviours can be an effective strategy to deter predators, and highly aggressive individuals or species may experience lower predation pressure than their close relatives (Elliott 1986, Lahti, Laurila et al. 2001, Conrad, Weinersmith et al. 2011). For instance, stickleback fish in environments under high predation pressure are more aggressive towards conspecifics and

show more pronounced anti-predator behaviours compared to populations that experience low predation pressure (Bell 2005). On the other hand, cichlids (*Neolamprologus pulcher*) that spend less time standing vigil (passive anti-predator behaviour) than their conspecifics are consistently more aggressive towards predators (Hess, Fischer et al. 2016). Similarly, defending brood (parental aggression) can be beneficial as a form of inclusive fitness (Pressley 1981). In species that show parenting behaviour, it is common for parents to become highly aggressive when accompanied by brood. Animals who would normally flee from disturbance when alone may aggressively initiate fights when perceiving a threat to their offspring; for instance, sows (*Sus scrofa*) become "increasingly protective and unpredictable" at the later stages of pregnancy (Graves 1984), and react aggressively when exposed to vocalization of distressed piglets, even if unrelated to them (Dellmeier and Friend 1986).

Non-defensive aggression

Aggression may also manifest in animals outside of self-preservation contexts. Competition pressure is a strong cause of inter- and intraspecific aggression in animals. When environmental resources are scarce, being able to successfully fight off competitors can be highly adaptive (Sih, Bell et al. 2004a, Sih, Cote et al. 2012). Aggression is well understood within the context of competition for food (e.g. Maupin and Riechert 2001, Pintor and Byers 2015), access to mating (e.g. Clutton-Brock, Albon et al. 1979; also see Darwin 1871, Lindenfors and Tullberg 2011), and territoriality (e.g. Riechert and Hendrick 1993, Duckworth 2008). A more recent and integrative approach to the role of aggression in competition is when it happens in animal societies in the form of hierarchical disputes. More aggressive, higher ranking individuals often have prioritized access to food, shelter, and reproduction (Drews 1993, Herberholtz, McCurdy et al. 2007).

Aggression can also play a decisive role for the acquirement of resources in non-competitive contexts. In predatory animals, being able to subdue prey is fundamental to survival. Consistently aggressive animals can be better fighters and achieve higher success in successfully hunting prey (Riechert and Hendrick 1993, Weinshenker and Siegel 2002, Michalko and Pekár 2017).

The costs of aggression

Aggressiveness as a behavioural trait is not always beneficial, however. Confrontations are risky for the animals involved and may result in mutual losses (Geist, 1974). Even if there is no direct physical aggression between participants, aggressive displays can be energetically costly (Caryl 1981). From a fitness perspective, aggression must occur under essential circumstances; in other words, the benefits of winning an aggressive contest should outweigh the costs of losing it (Maynard-Smith 1976, Georgiev, Kimczuk et al. 2013). Indeed, contests between animals are often resolved in non-contact displays before they escalate to actual fighting (Caryl 1981). Furthermore, because aggression in animals is rarely fully plastic and aggression tends to be a consistent trait (DeWitt, Sih et al. 1998, Sih, Bell et al. 2004a), aggressiveness can be non-adaptive when misdirected. For instance, individuals that exhibit consistent aggressive behaviour can also exhibit poor parental care (Sih, Bell et al. 2004a).

1.2 AGGRESSION BEHAVIOURS IN SOCIAL INSECTS: AN OVERVIEW

1.2.1 Eusociality and Superorganisms

Eusociality in insects

"Social insect" is a term commonly used to indicate a polyphyletic grouping of eusocial insect species (Batra, 1966). Historically, the term referred to societies of Hymenoptera (social wasps, ants, and social bees) and termites (Wilson, 1971). More recently, as eusociality has been described in other taxa, "social insect" may also refer to particular species of beetles, gall-inducing aphids, thrips, and others (Crespi, 1992; Kent & Simpson, 1992; Stern, 1994).

Although many animal species are capable of living collectively, "true societies" are a much rarer evolutionary phenomenon. Eusociality is defined as the highest level of social organization, where eusocial species exhibit (1) cooperative brood care, (2) overlap of adult

generations, and (3) reproductive division of labour (Michener, 1969; Wilson, 1971). The collective formed by individuals in a society and their nests is referred to as a "colony".

Colony cycles

The life history of social Hymenoptera colonies occurs within cycles, which functions akin to physiological ageing in unitary organisms (Hölldobler and Wilson 2008). Cycles vary in length and phases taxonomically and biogeographically; for instance, temperate species of social wasps often have more synchronous and well-defined cycles due to seasonal patterns of climatic and food availability variation (Ross and Matthews 1991), while ant colonies in desert environments can survive for many years regardless of seasonality (Gordon 1999). A general hypothetical model for a social insect colony cycle can be divided into four phases: (1) founding, when colonies are smallest, and the queen is laying eggs that will hatch into the first generations of workers; (2) ergonomic, when multiple generations are present and colony activity and brood population are at its maximum; (3) reproduction, when colonies are producing individuals for mating (gynes and males); and (4) senescence, often following queen death, when populations diminish and the colony usually collapses (Wilson 1971) (Fig. 1.1). The transition between phases of the cycle is followed by dynamic shifts in the demographical composition of its populations, which in turn result in different physiological needs for the colony (London and Jeanne 2003). In response, colonies at different stages will often show significantly different behaviours to cater to those needs.

Selection at the colony level

The life history of eusocial insects – with special regards to their reproduction – means that a colony, in addition to its individual members, is the primary unit upon which evolutionary pressures act (Wilson, 1985; Korb and Heinze, 2004). We can monitor behaviour at both the colony level, as well as the individual level in those colonies (Keller, 1999). Behavioural investigations at the colony level are especially interesting since they may provide key insights on the colonies' fitness and survival, being a cornerstone to

understand social hymenopteran evolution (Sih, Bell et al. 2004; Jandt, Bengston et al. 2014; Wright, Lichenstein et al. 2019).

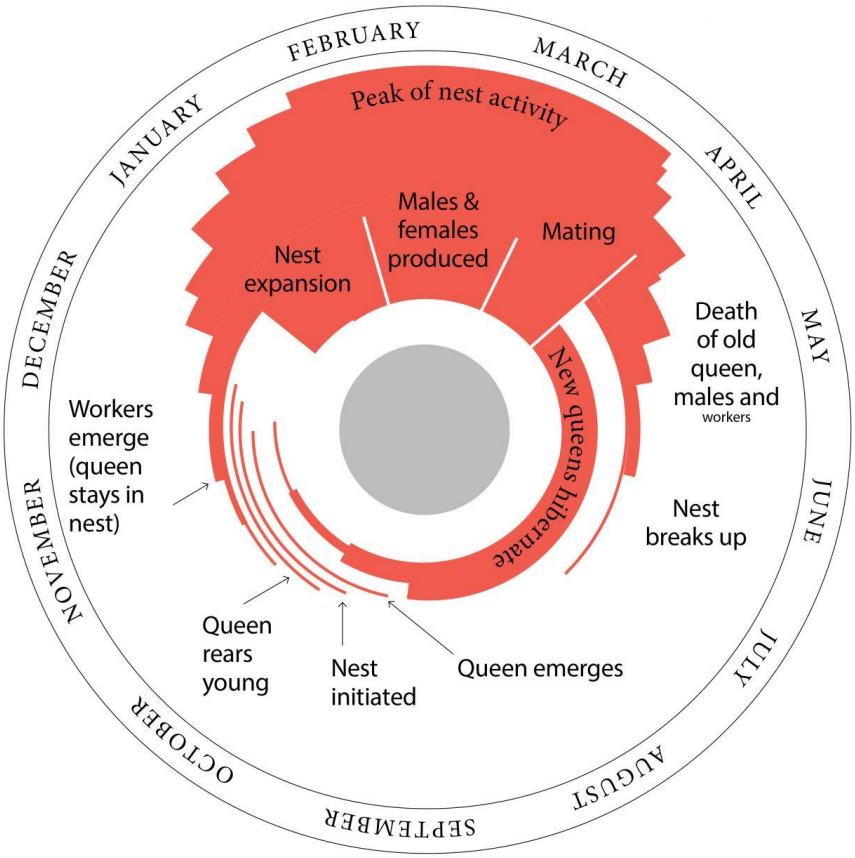

Figure 1.1. Colony cycle of *Vespula* wasps, a social insect, in New Zealand around the year (as originally published in Hansford, 2017; included with permission of original author Manaaki Whenua - Landcare Research / Henrik Moller). *Vespula* populations show synchronous seasonal colony cycles typical to social insects in temperate areas.

1.2.2 Aggression and the Ecology and Evolution of Eusociality

Aggression and eusociality

Social insects – notably wasps – are well known for their ability to aggressively defend their nests (Sumner, Law et al. 2018). It stands to reason that defensive and paternal aggression have an important ecological role for social wasps, but other types of aggression

(competition, social conflict, and predator) are also present in the group. This implies there must be great evolutionary value attached to aggression in its different forms. While cooperative behaviours (such as food sharing in communal nesting species; Amdam, Csondes et al. 2006, Toth, Varala et al. 2007) may be important factors driving the evolution of eusociality in Hymenoptera, aggressive behaviours also may have a key role in the process (Rittschof and Grozinger 2021). It is even possible that benefits of maternal aggression, including in early co-nesting species, are more relevant than other cooperative behaviours (e.g. collective food provisioning) to determine fitness (Strassmann 1981, Strassmann, Queller et al. 1988, Wong, Meunier et al. 2013).

Aggression and the social hierarchy within colonies

As insect societies evolved from their solitary ancestors (Rehan and Toth 2015), the division of reproductive labour became more distinct (Brian 1979). Social insect colonies can have from one (monogynous) to multiple (polygynous) individuals devoted specifically to reproduction ("queens") (Hölldobler and Wilson 1977, West-Eberhard 1978, Keller 1993, Hayo, Velthuis et al. 2006), and in most species workers also retain the ability to lay eggs (Bourke 1988). Colonies with multiple reproductive females often experience reproductive competition, usually in the form of a queen (or queens) trying to ascertain monopoly over reproduction (Olejarz et al. 2017). In some species of social Hymenoptera and termites, top-down enforcement of reproductive exclusivity can be achieved through chemical communication (Keller and Nonacs 1992, Kocher and Grozinger 2011). For instance, pheromones produced by queens are suggested to be involved in the inhibition of worker reproduction in *Bombus terrestris* bumble bees (Alaux, Sinha et al. 2004, Lopez-Vaamonde, Brown et al. 2007).

Paper wasps (Hymenoptera: Polistinae) are useful models to study the role of aggression in competition for reproduction (Jandt, Bengston et al. 2014). A *Polistes* colony is often founded by multiple reproductive females which, typically after worker emergence, engage in a series of aggressive interactions (e.g. biting or grappling) to determine the social hierarchy structure (West-Eberhard 1969); dominant females then become the primary reproductive individual in that colony. Subordinates may be killed or excluded from the

colony during these conflicts (Hughes and Strassman 1988, Reeve 1991). The structure remains stable through regular, ritualized aggression displays performed by dominant wasps and by the cannibalization of eggs laid by competitors, typically the subordinates (Jandt, Bengston et al. 2014, Olejarz, Allen et al. 2016). Similarly to paper wasps, aggressive interactions may play a vital role in asserting reproductive control in ants (e.g. *Leptopthorax gredleri*, Heinze, Lipski et al. 1992; *Veromessor pergandei*, Rissing and Pollock 1987) and bees (e.g. *Apis mellifera*, Gilley 2001; *Melipona beechei*, Jarau, van Vreen et al. 2010). A more extreme and particular example of the employment of aggression during reproductive competition can be seen in usurpers, where an unrelated reproductive is able to infiltrate a conspecific nest, kill the resident queen, and take over brood production while being aided by the dead competitor's worker offspring (Lorenzi and Cervo 1995).

Competitive aggression during foraging

Populous colonies need a significant and constant amount of energetic and nutritional intake (Wilson 1971, Detoni and Prezoto 2021). This means that, especially in environments where multiple colonies and/or species of social insects coinhabit, resource competition can be a main evolutionary pressure acting on colony survival (Holway, Suarez et al. 1998). Temporal resource partitioning, as seen in some desert ant species, is an example of a strategy used by foragers to avoid encounters with their competitors (Gordon 1999) while others may constantly relocate their colonies due to resource depletion (Tsuji 1988). On the other hand, direct confrontations may also occur in competing social insect foragers. When defending access to floral resources, some social bee species (*Trigona* spp.) engage in agonistic interactions towards interspecific competitors at the food source, ranging from aggressive displays to full-on deadly combat (Johnson and Hubbell 1974, Nagamitsu and Inoue 1997). Similarly, social wasps (*Agelaia* spp.) have been observed to engage in aggressive interactions with other wasps, as well as bees and ants, in baits placed in field experiments (Jeanne 1995).

Territorial aggression is another way in which competition involves direct confrontations. Ants may aggressively defend resource-rich territories around their nests (*Myrmecocystus mimicus*, *Oecophylla longinoda* and *Pogonomyrmex* spp.; Hölldobler and

Lumsden 1980); conversely, some species or colonies that are more prone to aggressiveness seem to neither achieve ecological dominance over more docile ones, nor to be more successful invaders in introduced ecosystems (von Aesch 2006). In fact, the opposite effect is often observed; colonies will often get desensitized by the presence of neighbouring foragers and display decreased aggression towards them, in what is known as the "dear enemy phenomenon" (Temeles 1994, Langen, Tripet et al. 2000, Dimarco, Farji-Brener et al. 2010).

Defensive aggression in social insects

The brood in a colony will make up future worker generations (colony growth and survival) and new reproductives (colony reproduction, Wilson 1971). While brood (eggs, larvae, and pupae) are virtually defenceless against predators, a large brood mass constitutes an attractive and nutritious resource for potential predators (Spradbery 1973, Ying, Xiaoming et al. 2010). This predation pressure has been a major evolutionary force in the evolution of eusociality in Hymenopteran insects (Rittschof and Grozinger 2021), which is reflected in the diverse and sophisticated range of nest defence behaviours for which social insects are renowned (Schmidt 2016). At the colony level, nest defence is a form of defensive aggression that functions as a self-preservation strategy, but also analogously as maternal aggression in unitary organisms since it is dedicated to ensure the survival of the colony's brood (O'Donnell and Jeanne 2002, Rittschof and Grozinger 2021).

Another strong selective pressure on the survival of social insect colonies is seen in parasitoids (Schmidt-Hempel 1998). Parasitoidism is distinguished from classic predation as the parasitoids do not kill and eat their prey immediately; rather, they typically lay eggs on their hosts, and their once hatched offspring slowly consumes the host's tissues until its death (Godfray 1994). Despite the consequences of these relationships being less drastic over short periods, widespread parasitodism can have catastrophic effects for populations, possibly resulting in colony death (Schmidt-Hempel 1998). Defensive aggression has been suggested to play a role in preventing parasitoids attacks, similarly to what may happen when a colony faces predation. For instance, leaf-cutter ants (*Atta* sp.) show unique patrolling behaviours along their foraging trails; smaller workers will often "hitchhike" on larger foragers when

parasitoids phorid flies (*Neodohrniphora erthali*) are present, possibly to physically respond to flies trying to lay eggs on its nestmates (Vieira-Neto, Mundim et al. 2006). Although parasitoid-host relationships provide a rich avenue for exploring the ecological and evolutionary implications of aggression in social insects, they involve cues and biological mechanisms that significantly differ from those observed in prey-predator interactions (Eggleton and Belshaw 1992). Thus, I hereon focus the discussion on defensive aggression exclusively on anti-predator scenarios.

1.3 NEST DEFENCE IN SOCIAL INSECT COLONIES

1.3.1 Non-Aggressive Nest Defence

The social insect nest

The nest is the locus of social life for Hymenoptera societies (Starr 1991). Among the main benefits of living in a nest is the structural protection it offers against harsh environmental conditions (e.g. flooding, Kleineidam and Roces 2000; deadly temperatures, Gibo, Yarascavitch et al. 2012) and against predator attacks (Jeanne, 1975). There is incredible diversity in nesting site choices throughout the different social insect taxa. Nesting in cavities is likely the most widespread choice of site across different groups of social Hymenoptera; these can be underground (e.g. myrmicine ants, Wheeler and Rissing 1975; *Bombus* spp. bumble bees, Lye, Osborne et al. 2012; *Melipona* spp. stingless bees, Wille 1983; vespine wasps, Greene 1991, Matsuura 1991), or in natural, aboveground cavities such as tree hollows (e.g. many honey and stingless bees, Breed, Guzmán-Novoa et al. 2004, Roubik 2006; dolichoderine and myrmicine ants, Smallwood 1982, Hölldobler and Wilson 1990). Other common type of nesting site is seen in “aerial” nests built amidst vegetation, often benefiting from the structural complexity provided by branches and leaves (e.g. dolichoderine and pseudomyrmicine ants, Janzen 1973, Marini-Filho 1999; paper and vespine wasps, Gadagkar 1991, Jeanne 1991, Reeve 1991). Notably, army ants (Formicinae: *Eciton* spp.) and driver ants (Dorylinae: *Dorylus* spp.) do not build permanent nests at all, being largely nomadic (Hölldobler and Wilson 1990).

Nesting architecture similarly reflects the variety of behaviours observed taxonomically in social hymenopterans. Ant nest structure usually consists of a series of chambers assigned to different purposes (nurseries, food storage, etc.) interlinked by tunnels, which are linked to the nest's exterior through several openings (Trevis Jr. 1958, Wheeler and Rissing 1975). Nests of honey bees sometimes consist of exposed, stacked combs (Breed, Guzmán-Novoa et al. 2004), while paper wasp (Polistini and Mischocyttarini) nests are often a single uncovered comb (Jeanne 1975). Some species will also build protective structures incorporated to nests, such as envelopes that protect the combs (e.g. stingless bees, Rasmussen and Camargo 2008; vespine and Epiponini wasps, Jeanne 1975, Greene 1991, Matsuura 1991). The combination of both active and passive defence strategies has led to some authors comparing a social insect nest to a "fortress" (O'Donnell and Jeanne 2002) which is fiercely defended by its inhabitants.

Passive nest defence

As a rule, social Hymenoptera colonies usually rely on multiple defences against predators. Anti-predator behaviours can be "passive", rather than a responsive behavioural reaction to predation. For example, the number of potential predators can be reduced simply by nesting out of most predators' reach, such as seen in *Apis dorsata* honey bees nesting on the top of tree crowns (Seeley, Hadlock Seeley et al. 1982), or by building cryptic nests that blend into the environment to avoid predator attention (e.g. small, camouflaged nests of *Mischocyttarus iheringi* paper wasps; Barbosa, Maciel et al. 2016). Some species associate themselves to other, more aggressive organisms that can offer some protection; for instance, stingless bees often nest near ant, bee, wasp, and termite colonies (Roubik 2006). Other passive defences come into play during the predator attack: turtle ant (*Colobopsis truncata*) soldiers block nest entrances against non-nestmates (Forel 1874), while *Polybia emaciata* wasps invest in structural damage resistant nests by building them of hardened mud rather than vegetal fibre (O'Donnell and Jeanne 2002).

1.3.2 Dedicated Defenders

The sting of social insects

All female Hymenoptera have a stinging apparatus; parasitoids use it to lay eggs, hunters use it to subdue their prey, and social insects use it for predator defence (Shing and Erickson 1982, Schmidt 2016). The stinging apparatus and its associated venom glands have probably evolved from the female's ovipositor first in a solitary wasp ancestor, which used it to paralyze prey (Schmidt 2004). In advanced insect societies, stinging is a highly effective anti-predator defence, especially when performed cooperatively by nestmates, and is widespread in different taxa (Starr 1985, Nouvian, Reinhard et al. 2016). The venom injected may kill or incapacitate predators. Even when that is not the case, the pain induced may enforce a negative association of social insects with negative experiences for a predator (Schmidt 2016). Further reinforcement can be provided by visual cues, such as the aposematic coloration in some bees and wasps (Vidal-Cordero, Moreno-Rueda et al. 2012). This may result in long-term fitness gain for insect colonies, in addition to the short-term advantages of deterring a predator.

Guards and soldiers

The division of labour during nest defence within insect colonies happens at varied degrees of behavioural adaptations. Workers that consistently display nest defence behaviours have been described as belonging as "guards" and "soldiers" (or "stingers", Nouvian, Reinhard et al. 2016). In many species, these groups are monomorphic; in other words, they do not differ morphologically from other workers. Guards usually sit at the nest entrance, inspecting incoming foragers and are the first workers to react to a non-nestmate's presence (Breed and Rogers 1991, Paxton, Sakamoto et al. 1994, O'Donnell, Hunt et al. 1997, O'Donnell and Jeanne 2002). The guard subcaste is seen as a transitional phase for workers between in-nest and external task allocation (Moore, Breed et al. 1987), and is usually comprised of a very small number of individuals – although colonies under higher predation pressures may allocate more workers to guarding efforts (Breed, Smith et al. 1992, Guzmán-

Novoa, Hunt et al. 2004). Soldiers, on the other hand, are alerted by the guard's behaviour, and are ultimately responsible for aggressive fights with predators (Breed, Robinson et al. 1990, Breed, Smith et al. 1992). In aerial, enveloped nests of swarm-founding paper wasps, colonies may keep a number of "night guards"; even when the colony is not active at night, groups of workers will still stay on the outside of the nest in order to react to predator attacks more effectively (Chavaría-Pizarro and West-Eberhard 2010).

Polymorphism and size differences can also drive division of labour in social insects, notably ant species (Westwood 1838). These ant "soldiers" are on average larger than their nestmates (Wilson 1982, Whitehouse and Jaffe 1996), and have a range of morphological adaptations for nest defence. The typical polymorphic ant soldier has a proportionally enlarged head with powerful mandibles which are used to cut (e.g. Myrmicinae: *Atta* spp., Whitehouse and Jaffe 1996) or pierce (e.g. Formicinae: *Eciton* army ants, Chadab 1979a) their targets. Some particularly extreme morphological adaptations for nest defence can be seen in mandibles that have been modified to launch small invertebrates away from the nest (Myrmicinae: *Orectognathus versicolor*, Carlin 1981). Morphological differentiation of subcastes also occur in some Meliponini stingless bees (Grüter, Menezes et al. 2012, Grüter, Segers et al. 2017), despite being absent in most other bee and wasp species.

Temporal polyethism and nest defence

Most social Hymenoptera show some degree of "temporal polyethism", where task partioning is determined by the worker's physiological age (Wilson 1968, Winston 1987, Hurd, Jeanne et al. 2007). For instance, in honey bees (Hymenoptera: Apidae), younger workers usually perform in-nest tasks such as nursing, while older bees specialise in foraging and nest defence (Winston 1987). It is hypothesized that, as individuals age, they become "expendable" to the colony (Ahn, Xie et al. 2012). The relationship between older age and performing out-of-nest tasks, which include nest defence, seems to be near ubiquitous in Hymenoptera and is likely to be the main mechanism at work to determine dedicated nest defence tasks, especially in monomorphic species where guards and soldiers do not differ morphologically from their nestmates.

1.3.3 Collective Nest Defence

Defence strategies and nest structure

Because the nest acts as a buffer between a colony and its external environment, effective nest defence must be adequately adapted to the nest's physical structural organisation. In ant colonies, which tend to be much bigger and more populous than most bee and wasp ones, defences against predators occur primarily at the nest entrance and peripheral tunnels, where "fighting balls" may form – masses of soldiers ready to respond to a predator's attack (Whitehouse and Jaffe 1996). However, if a portion of the nest collapses due to predation from a large animal (e.g. in earthen mounds of *Solenopsis* fire ants (Myrmicinae); Tschinkel 2006), the colony needs to be able to effectively relocate its defenders away from the openings and onto the disturbed site (Haight 2010). Bee and wasp colonies that live in sheltered or enveloped nests typically place guards at their entrances (Moore, Breed et al. 1987, Chavaría-Pizarro and West-Eberhard 2010). In species without these protective structures, defensive efforts need to be adapted to the more diffused exposure to external cues. For instance, in some honey bee nests, "sheets" of guards will cover the external surfaces and monitor incoming predators (Breed, Guzmán-Novoa et al. 2004).

Nesting site also plays a role in which defensive strategies colony guards must use in order to effectively react to a predator attack. For instance, in aerial paper wasp nests, especially those without protective envelopes, predator detection may occur primarily via visual cues since wasps are constantly and directly exposed to the environment outside the nest (Furuichi and Kasuya 2013). Conversely, in underground nesting species, chemical perception (e.g. volatiles in the predator's breath and dispersal of alarm pheromones within the nest; McCann, Moeri et al. 2015, Jandt, Detoni et al. 2020) may be a more effective mechanism to ensure the colony's fast reaction to disturbance.

Defences against vertebrate predators

Large vertebrate predators (e.g. birds and mammals, including humans) figure amongst common threats faced by social insect colonies (Jeanne 1975, Whitehouse and Jaffe

1996, Nouvian, Reinhard et al. 2016). The predator's presence is perceived by the colony through visual, chemical, or vibroacoustic cues (Jones, Clark et al. 2004, Fujiwara-Tsujii, Yamagata et al. 2006, Haight 2010, McCann, Moeri et al. 2015, Jandt, Detoni et al. 2020). In some bees and wasps, before the actual stinging takes place, some guards will fly at and around the predator (Collins, Rinderer et al. 1980); this functions essentially as an aggressive display, since very few guards actually engage in direct aggression (Cunard and Breed 1998). Other guards will stay inside the nests and produce alarm pheromones, which are then fanned towards their nestmates (Collins, Rinderer et al. 1980). This chemical communication alerts soldiers (and other workers) of the intruder's presence, to which they react by flying out of the nest and often executing collective stinging behaviour (Wagner and Breed 2000). Soldiers and other workers defend the colony by holding on to a vertebrate invader with their mandibles while delivering stings, which range from a single to a dozen per individual across taxa (Wagner and Breed 2000, Postma 2006). The sting injects not only venom, but also more alarm pheromones onto its target (Hermann 1971); this both elicits a painful reaction in the target and helps continually recruit more defenders to attack it. In some species, such as some large bodied ant species (Paraponerinae: *Paraponera clavata;* Ponerinae: *Dinoponera*), the venom is so powerful that a single sting from a soldier can incapacitate a predator as big as a human (Haddad Junior, Cardoso et al. 2005, Schmidt 2016).

Stinging is not the sole strategy employed during colony defence. In many species, ant soldiers' primary form of defence against large vertebrates is using their mandibles for biting (Hölldobler and Wilson 1990, Whitehouse and Jaffe 1996). In large numbers, the pain caused by tens to hundreds of bites may be enough to drive a predator away. A clear example of this is seen in leafcutter ants (Mymicinae: *Atta*), in which the enlarged mandible sizes of soldiers can cut through the thick cuticles of large vertebrates (Whitehouse and Jaffe 1996). Some bees species who are unable to sting, such as the aptly named stingless bees (Hymenoptera: Apidae: Meliponini), instead react aggressively by biting and using excreted resin-like substances to attach themselves to the predator or to immobilize it (Nunes, von Zuben et al. 2014).

Defences against invertebrate predators

Invertebrates are common predators of social insect colonies – often being social insects themselves, such as rival ant and hornet colonies (Chadab 1979b, Ono, Igarashi et al. 1995). Because invertebrate attacks differs from a vertebrate's in that the predator is smaller, has a chitinous cuticle, and often attacks in large numbers defences that are effective against vertebrates (e.g. collective stinging) are often not effective in deterring invertebrates. A raid from a rival colony can lead to the prey colony's failure (Chadab 1979b), and therefore some social insect species have developed a highly specialized set of anti-invertebrate aggressive behaviours.

Both bee and wasp colonies have to deal with social insect predators (Matsuura and Sakagami 1973, Nouvian, Reinhard et al. 2016). Aggressive anti-ant defences are rarely observed, but sometimes workers will use high-frequency wing-buzzing to push ants off the nest entrance (Chadab 1979b, Jeanne 1991, Yang, Radloff et al. 2010). A unique case of specialized behaviour is seen in the Japanese honey bee (*Apis cerrana*) against hornet intruders, named thermal defence behaviour. A mass of workers forms around the invader and, by vibrating their abdomens together, they are able to locally raise the temperature up to deadly levels for both the defenders and the hornet (Ken, Hepburn et al. 2005). Although this seems to be a drastic and costly effort, killing hornet scouts will prevent them from recruiting their own nestmates to raid the bee hive; a collective hornet raid can easily result in the death of the whole colony (Matsuura and Sakagami 1973). Similar phenomena occur for stingless bee colonies targeted by intraspecific usurpers and intraspecific bee raiders; they will try to eliminate scouts by biting them to death them or chewing their wings off, in a way that the scouts are unable to leave the foreign nest and recruit their nestmates back to the food source (Grüter, Menezes et al. 2012). Another fascinating adaptation of defensive aggression is seen in the "exploding ant" (Fomicinae: *Colobopsis saundersi*), which reacts to the presence of arboreal invertebrate predators (including other ants) near their nests by bursting their abdomens and showering their targets in corrosive chemical concoctions (Jones, Clark et al. 2004). The execution of this behaviour, "autothysis", is deadly to the defender, but often succeeds in deterring invaders from preying upon the colony.

1.4 THE DRIVERS OF DEFENSIVE AGGRESSION IN SOCIAL INSECTS

1.4.1 Consistent Aggressive Behaviours

Behavioural consistency in animals

Aggression in animals can often be studied in the context of behavioural consistency (Sih, Bell et al. 2004a, Jandt, Bengston et al. 2014, Wright, Lichenstein et al. 2019). Historically, animal behaviour was studied with the assumption of plasticity; in other words, animals were expected to optimally modulate their behavioural responses to environmental cues in an adaptive way (Scheiner 1993). In these hypothetical systems, consistent behavioural variation was interpreted as non-adaptive, or as statistical noise (Sih, Cote et al. 2012).

Currently, the field of behavioural ecology acknowledges limited behavioural plasticity as being the norm, rather than the exception (Sih, Bell et al. 2004a). Plasticity is constrained by a number of biological processes in animals (e.g. morphological and cognitive limitations, Hazlett 1995), which may explain its rarity in systems. On the other hand, consistent individual differences (CIDs) in behaviour are common, which means some animals may act in ways that can be considered non-adaptive or maladaptive in some ecological contexts (Wilson, Clark et al. 1994). There is plenty of evidence that maintaining a suite of CIDs at the population level can have important fitness consequences for a species (Sih, Cote et al. 2012). A consistent behavioural profile (such as "aggressive" or "docile") that is observed over time or across ecological contexts is named a behavioural type (or "personality") (Sih, Cote et al. 2012, York 2018).

"Aggressive" vs. "Docile" behavioural types

"Aggressive" animals are more likely to engage in aggressive confrontations with predators, whereas "docile" ones tend to adopt other defensive strategies, such as fleeing (Sih 2004). In the social Hymenoptera, aggressive behavioural types have been suggested to underlie behavioural castes (Jandt, Bengston et al. 2014). In monomorphic colonies, it has

been demonstrated that older individuals usually show “aggressive” behavioural types, versus the “docile” types shown by younger ones (bees, Seeley 1982, Rittschof, Coombs et al. 2015; wasps, O'Donnell 2001, Santoro, Hartley et al. 2015, Santoro, Hartley et al. 2019).

Colony behavioural types

For social insects, consistent defensive aggression can also vary collectively (Jandt, Bengston et al. 2014). A classic example of this is seen in honey bees (Apidae: *Apis mellifera*), where colonies of the “Africanized” lineage tend to be consistently more aggressive when disturbed when compared to “European” lineages (DeGrandi-Hoffman, Collins et al. 1998). Wasp (Vespidae: *Vespula* spp.) colonies may also show consistent aggressive phenotypes, in which anti-predator aggression varies consistently both within and across species in the same genus (Jandt, Detoni et al. 2020). In some invading ant species, aggressive colony types have been positively correlated to ecological success in displacing competitors (Davidson 1998, Rowles and O'Dowd 2007).

1.4.2 Social Environment

Colony demography

A straightforward relationship between colony demography and defensive aggression has been observed in African honey bee (*Apis mellifera scutellata*) colonies: bigger hives are able to employ more guards and soldiers, and thus show increased aggressive response when disturbed (Schneider and McNally 1992). Similarly, bigger colonies of the *Leptothorax ambiguus* ant are more aggressive towards non-nestmates than smaller ones (Stuart 1991). However, this is not always the case, as defensiveness in insect colonies can be decoupled from colony size (Jandt, Detoni et al. 2020). An important drive for aggressiveness towards predators seems to be the ratio between brood and workers in a colony. Higher brood populations directly represent the colony’s reproductive investment, which acts as a motivator for increased nest defence in the proportionally smaller adult population (London and Jeanne 2003). There is evidence that colonies in the ergonomic phase – when brood

population is highest – are consistently more aggressive than colonies in other stages of the development cycle (Judd 1998, London and Jeanne 2003, Monceau, Bonnard et al. 2013).

1.4.3 Colony Health

Parasitism and disease

Parasites and disease vectors often induce physiological shifts in their animal hosts, which may then result in behavioural changes (Kavaliers, Colwell et al. 2000). Because of their organisation in colonies, social insects are especially vulnerable to widespread parasitic infections (Schimdt-Hempel 1995). Heavily infected colonies may experience significant changes in their behaviours, which are often impaired by the parasite's action (Schneider and Drescher 1987). For instance, honey bee (*Apis mellifera*) foragers infected by the parasitic mite *Varroa destructor* show decreased flight manoeuvrability when compared to healthy conspecifics (Mujires, van Dooremalen et al. 2020).

Parasites might accelerate age polyethism in bee colonies, which in turn might increase the prevalence of aggression-prone workers (Lecoq, Jensen et al. 2016); furthermore, it is possible that viral infections stimulate aggression behaviour in soldiers (Fujiyuki, Takeuchi et al. 2004). Increase in aggression in parasitized colonies might represent an adaptive response to infection, as aggression towards diseased nestmates may help control further spread of parasites within colonies (Cremer, Armitage et al. 2007, Drum and Rothenbuhler, 1995). Other forms of aggression such as aggressive defence of foraging territories can reduce contact between neighbouring colonies and help prevent horizontal parasitic spread in social insect populations (Boomsma, Schmidt-Hempel et al. 2005). Supporting evidence of the role of aggression in controlling disease can be seen in honey bees infected by the parasitic fungus *Beauveria bassiana* (Cappa, Petrocelli et al. 2019). Diseased individuals have been shown to have altered cuticular hydrocarbon profiles, which reduces conspecific aggression and facilitates their entry in neighbouring colonies – potentially helping in the spread of the pathogen (Cappa, Petrocelli et al. 2019). In addition, preliminary observations suggest that widespread viral infections in dense populations of

social wasps (*Vespula* spp.) may reduce defensive aggression at the colony level (Phil Lester, personal communication).

1.4.4 Other Environmental Influences

Abiotic factors

Abiotic variables (e.g., temperature, air humidity, wind speed, luminosity) affect the behaviour of the colony as a whole (Huffaker and Gutierrez 1999, Raveret Richter 2000). Because nest defence occurs at the nest's periphery, it is expected that these environmental factors may play a part in the variation of aggression. However, few studies have found a significant relationship between colony aggression and abiotic variables. Honey bee (*Apis mellifera*) colony defence is most aggressive in high temperatures and humidity, and when wind speeds are low (Southwick and Mortitz 1987). A possible explanation for this is that although these conditions allow for unhindered flight, high humidity means that most foragers – the older, more aggressive cohorts of workers – are inside the nest, and thus the colony is more likely to respond intensely to a predator attack. Conversely, some species of *Temnothorax* ants show decreased aggression in consistently warmer areas (Segev, Burkert et al. 2017, Horna-Lowell, Neumann et al. 2021). Because abiotic variables are often intrinsically related to other causal mechanisms underlying aggression, such as food availability (see below), identifying and explaining their effect on nest defence can be particularly challenging.

Food availability

The availability of food can moderate competitive, and indirectly defensive, aggression behaviour in social insects. Environmental food shortage can be correlated with increased colony raiding and honey robbing in bees (Rittschof and Nieh 2021), which in turn increases nest guarding efforts (Downs and Ratnieks 2000). Food availability has been linked to shifts in nest defence efforts across some social insect taxa. Lab-reared yellow crazy ants (*Anoplolepis gracilipes*) were overall more active, and more aggressive towards opponents,

when provided with greater food sources (Wittman, O'Dowd et al. 2018). *Messor andrei* black harvester ants also show increased activity in response to abundant food, but instead become less reactive to alarm pheromones (Pinter-Wollman, Gordon et al. 2012). Similarly, *Apis mellifera* honey bees are more acceptant of non-nestmates when food is abundant (Downs and Ratnieks 2000), which is supported by the fact that older (and thus more aggressive) individuals have lower physiological energetic reserves (Toth and Robinson 2005). In fact, starvation may accelerate age polyethism in honey bees altogether.

1.4.5 Experience

Habituation and sensitization

In social insects, cognitive processes are often involved in short and long term shifts in behaviour (Chittka and Muller 2008, Avarguès-Weber, Deisig et al. 2011). A classic example is associative learning in foragers, which are able to finely associate environmental cues to rewards at varying quality levels (Waser 1986). Defensive aggression is an ideal model for studying learning processes in individuals; since anti-predator behaviours can be energetically costly to execute and often involve risks to the defender (Rittschof and Grozinger 2021), fine tuning aggressive responses based on past experiences should be positively selected in insect societies.

Experience can modulate aversive response in social insect individuals in varying ways. For instance, ants (*Atta colombica* and *Linepithema humile*), honey bees (*Apis mellifera*) and common wasps (*Vespula vulgaris*) have been shown to heighten responsiveness to aggressive stimulation in controlled conditions with increasing experience (Roussel, Carcaud et al. 2009, van Wilgenburg, Clémencet et al. 2010, Santoro, Hartley et al. 2015, Norman, Pamminger et al. 2017). In a similar fashion, Argentine ants (*Linepithema humile*) who react aggressively to intruders may react more strongly to conspecific colonies whose worker's they are more exposed to (neighbours) than to ones they are not familiar with (Thomas, Payne-Marisâ et al. 2007). Conversely, the opposite phenomenon can occur: *Pheidole* ant workers that often come in contact with neighbouring foragers may habituate to their presence, resulting in lower aggression rates between neighbours (Langen, Triplet et al.

2000). Another example is seen in Asian needle ants (*Pachycondyla chinensis*), which may reduce their aggressiveness towards invading heterospecifics over repeated exposure (Rice and Silverman 2013), presumably to allow for feasible coexistence.

Colony learning

One of the consequences of the unique form of organisation in insect societies means that colonies, analogously to individuals, may also be capable of shifting behaviours based on previous experiences (Leadbeater and Chittka 2007). One mechanism suggested to explain how experience in individuals may translate into colony level is the social learning phenomenon (Leadbeater and Dawson 2017). Individuals might learn how to react to cues by observing or interacting with more experienced nestmates (Farina, Grüter et al. 2005, Avarguès-Weber, Deisig et al. 2018), which facilitates the horizontal spread of a behaviour across coexisting cohorts. If information gained from experiences is consistently shared between a significant portion of workers in a colony, the entire group might exhibit short-term shifts in behavioural phenotypes (Leadbeater and Chittka 2007). Long-term shifts in colony-level behaviour in response to consistent cues is an even more sophisticated phenomenon, possibly involving cross-generational transfer of information between workers (Kirchner 1987).

There is some evidence that colonies, like individuals, can learn from experiences where defensive aggression is at play. Honey bee (*Apis mellifera*) colonies can habituate to non-threatening experimental disturbance events, reducing defensive aggression over time (Rittschof and Robinson 2013, Rittschof 2017). Similar phenomenon may also occur, at least in short spans of time, in colonies of *Vespula* wasps (see supplement in Jandt, Detoni et al. 2020) and stingless honey bees (*Tetragonisca angustula*, Jernigan et al. 2018). Conversely, defensive aggression in some insect societies can be largely driven by intrinsic factors, with experience seemingly playing no role (*Polybia occidentalis* paper wasps, London and Jeanne 2003).

1.4.6 Physiology

Juvenile Hormone

Juvenile Hormone (JH) has a pivotal role in insect growth and development (Riddiford 1994). JH levels have been shown to underly the variation of a wide range of behaviours throughout maturation processes (e.g., Webster and Cardé 1984, Tojo, Morita et al. 1985, Meunier, Belgacem et al. 2007). Typically, JH production is highest early in life, decreasing steadily as insects become adults (Riddiford 1994); in holometabolous insects such as the Hymenoptera, a sharp decrease in JH production drives metamorphosis from larva into pupa and pupa into adult (Konopova, Smykal et al. 2011). Still, spikes in the production of JH in adult brains can be traced to behavioural responses (Tibbetts and Huang 2010, Shpigler, Saul et al. 2017). In honey bees, JH titers are involved in age-related task division and the transition from internal into external nest behavioural suites (Sullivan, Jassim et al. 2000, Elekonich, Schulz et al. 2001, Whitfield, Cziko et al. 2006), which possibly would include nest defence. In *Polistes* workers (Vespidae: Polistinae), reproductive conflict and increased nest guarding coincides with upregulated JH titers (Giray, Giovanetti et al. 2005, Tibbetts and Huang 2010). Specifically for defensive aggression, it has been suggested that JH may also play a role in anti-intruder behaviours, with honey bee guards showing high JH production (Huang, Robinson et al. 1994, Hartfelder 2000).

1.4.7 Genetics

Aggression genes

Genes encoding aggression behaviours are probably highly conserved in insect evolution, from solitary taxa such as *Drosophila* flies to the eusocial Hymenoptera (Toth, Varala et al. 2010). However, the majority of current studies linking genetics to aggression focus on the competitive and social aspects aggression, rather than defensive aggression (e.g. Suarez, Holway et al. 2008, Toth, Varala et al. 2010).

An important exception are investigations with honey bee population genetics. After consistent colony defence phenotypes across *Apis mellifera* lineages were initially described (DeGrandi-Hoffman, Collins et al. 1998), cross-breeding experiments and molecular evidence have showed that the variation in defensive aggression is not only heritable, but can also be studied at the transcriptomic level (Giray, Guzmán-Novoa et al. 2000, Guzmán-Novoa, Hunt et al. 2004, Guzmán-Novoa, Hunt et al. 2005, Alaux, Sinha et al. 2009, Rittschof and Robinson 2013, Avalos, Fang et al. 2020). Alaux, Sinha et al. (2009) showed that typically aggressive Africanized honey bees, when reared by more docile European colonies, still retain a relatively more aggressive phenotype than their nestmates, which can be measured in differentially expressed brain genes.

Other molecular factors of defensive aggression

Although demonstrations of a direct link between genes and defensive aggression are scarce in social insect literature (Rittschof and Grozinger 2021), molecular processes that have been shown to play a role in aggression-related aspects of aculeate biology can be used to make inferences on the role genes may play in individual and colony phenotypes. Such is the case for studies that focus on genetic differences between a colony's guards and its other worker subcastes (Breed, Robinson et al. 1990b, Page and Robinson 1991, Smith, Toth et al. 2008). Genes that play a role in the determination of age-based division of labour – e.g., those that are differentially expressed in older individuals in contrast with younger ones (ants: Ingram, Oefner et al. 2005, Lucas, Hughson et al. 2010, Oettler, Nachtigal et al. 2015; bees: Toma, Bloch et al. 2000, Ben-Shahar, Robichon et al. 2002, Kucharski and Maleszka 2002, Whitfield, Cziko et al. 2003, Whitfield, Ben-Shahar et al. 2006) – are good candidates to provide a base for further investigations of the genetic bases of defensive aggression in insect societies. This is further reinforced by the fact that aggression and foraging in honey bees (both typically performed by older workers) share metabolic pathways in the brain (Rittschof, Vekaria et al. 2018, 2019).

1.5 OBJECTIVES AND GOALS

1.5.1 Thesis Aims

General goal

Defensive aggression against predators has played a major role in the evolution of eusocial Hymenopteran groups (Rittschof and Grozinger 2021). The aggression behaviours of social insects are often expressed consistently at both individual and colony levels (Jandt, Bengston et al. 2014, Wright, Lichenstein et al. 2019), although the mechanisms that explain this consistency differ across taxa and ecological contexts (Bengston and Jandt 2014). In this thesis, I studied defensive, anti-predator aggression behaviours in colonies of the social wasp *Vespula vulgaris* in New Zealand. By describing the behaviour of *V. vulgaris* during nest defence, as well as investigating different molecular, ecological, and evolutionary mechanisms that might help explain the rise of aggressive phenotypes in the species, I contribute to the general understanding of the evolution and maintenance of eusociality in animals.

Study system

Vespula vulgaris was first identified in New Zealand in 1921, possibly after an accidental introduction from its native range in Europe (Lester, Gruber et al. 2014, Lester and Beggs 2019). The lack of native predators has lead *V. vulgaris*, alongside with its congeneric and co-invader *V. germanica*, to successfully establish itself in the country in what is probably the most successful case of a social wasp biological invasion recorded in human history (Lester 2018). Colonies of the species are widespread throughout New Zealand territory. In especially vulnerable areas, such as the beech forests of the Nelson Lakes region of the South Island, *Vespula* can reach an incredible average of 12 colonies per hectare (versus the typical 0.4 nest per hectare they show in their native range; Barlow, Beggs et al. 2002). These abnormally dense populations have a catastrophic effect on the native fauna and microbiota due to overpredation and competitive exclusion by wasp foragers

(Lester 2018). Additionally, invasive wasps impact the nation's economy in an estimated 130 million of New Zealand dollars per year, spread across medical, agricultural, and pest control expenses (MacIntyre and Hellstrom 2015).

One of the few positive outcomes of the *Vespula* invasion in New Zealand is the fact that increased colony population density allows for readily available samples for field-based aggression behaviour studies. In fact, Jandt, Detoni, and colleagues demonstrated that *Vespula* colonies show significant variation in their aggressive behavioural phenotypes within and across species (Jandt, Detoni et al. 2020). This consistent variation, in addition to the existence of accessible methods to observe and quantify *Vespula* aggression in the field (McCann, Moeri et al. 2015), makes *Vespula* an ideal study system to investigate the mechanisms underlying behavioural variation.

1.5.2 Specific Questions[1]

Who are the predators of social wasps?

The existence of noteworthy defensive aggression in social wasp colonies suggests the presence of strong and frequent predation pressures at ecological and evolutionary scales. However, actual evidence of wasp colonies being preyed upon often is circumstantial (Birkhead 1974), and so far a comprehensive and taxonomically inclusive compilation of wasp predators worldwide has not been made available. In Chapter 2, I asked the questions: who are the predators of social wasps around the world? How do they vary according to prey taxa? How do wasps defend themselves against these predators? What can be inferred from prey-predator relationships for the evolutionary history of wasps?

Can nest defence in V. vulgaris *be predicted by colony age or experience?*

Colonies of social insects are able to modulate their behaviours over time based on their social environment and development cycles (London and Jeanne 2003), and sometimes also on collective experiences (van Wilgenburg, Clémencet et al. 2010). Building on previous evidence that *V. vulgaris* colonies have consistently different aggressive phenotypes in New

[1] The chapters in this thesis were formatted as to be submitted for publication as individual studies. Some repetition of information is to be expected.

Zealand (Jandt, Detoni et al. 2020), in Chapter 3, I asked the following questions: does a colony's nest defence change over time, and can it be predicted by experience with simulated predator attacks? Are there other environmental and intrinsic factors that can help explain variation in aggressiveness?

Do nest defenders show distinct brain gene expression patterns?

There is evidence that social insect aggressiveness may be driven by genetic mechanisms (Alaux, Sinha et al. 2009), and colony defenders often differ from other worker subcastes in their gene expression profiles (Giray, Guzmán-Novoa et al. 2000). In Chapter 4, I investigated the genetic basis for aggression in *Vespula* by asking the following questions: is there an "aggression gene" in *V. vulgaris*? Do nest defenders and foragers differ in their brain gene expression profiles?

Chapter 2

Evolutionary and Ecological Pressures Shaping Social Wasps Collective Defences[2]

2.1 ABSTRACT

Social insects are well known for their aggressive (stinging) responses to a nest disturbance. Still, colonies are attacked due to the high-protein brood cached in their nests. Social wasps have evolved a variety of defence mechanisms to exclude predators, including nest construction and coordinated stinging response. Which predatory pressures have shaped the defensive strategies displayed by social wasps to protect their colonies? I reviewed the literature and explored social media to compare direct and indirect (claims and inferences) evidence of predators attacking individuals and colonies of wasps. Individual foraging wasps are predominantly preyed upon by birds and other arthropods, whereas predators on wasp brood vary across subfamilies of Vespidae. Polistinae wasps are predominantly preyed upon by ants and Passeriformes birds, whereas Vespinae are predominantly preyed upon by badgers, bears, and hawks. Ants and hornets are the primary predators of Stenogastrinae colonies. The probability of predation by these five main Orders of predators varies across continents. However, biogeographical variation in prey–predator trends was best predicted by climate (temperate vs. tropical). In social wasps' evolutionary history, when colonies were small, predation pressure likely came from small mammals, lizards, or birds. As colonies evolved larger size and larger rewards for predators, the increased predation pressure likely selected for more effective defensive responses. Today, primary predators of large wasp colonies seem to be highly adapted to resist or avoid aggressive nest defence, such as large birds and mammals (which were not yet present when eusociality evolved in wasps), and ants.

Key words: aggression behaviour, nest defence, Polistinae, Stenogastrinae, Vespinae.

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2.2 INTRODUCTION

2.2.1 General overview

Social insects are perhaps best known for the fierce collective defence of their nests and the ability to deliver painful stings (e.g., *Pseudomyrmex* species [Hymenoptera: Formicidae] in ant acacias; Schmidt 2016, Sumner, Law et al. 2018). Wasps in particular have a special reputation for stinging; indeed, for millennia, human cultures throughout the world have taught their children to recognize the local wasp phenotype (e.g., the aposematic yellow and black stripes of Vespinae in temperate regions) and to fear and avoid social wasps (Lester 2018, Sumner, Law et al. 2018, Jones 2019). Despite widespread public recognition of the defensive talents of social wasps, we know remarkably little about the ecological and evolutionary forces driving the evolution of their defensive behaviours. The defensive behaviours of any organism evolve in response to selective pressures exerted by their predators; yet, little is known about the predators of social wasps (Fig. 2.1a). Although anecdotal reports are present in the literature, direct evidence of wasp predation is surprisingly scarce. To understand the (co)evolution of defensive behaviours in social wasps, we require a holistic understanding of who these predators are and to what extent these predator–prey relationships can be explained by ecological and/or evolutionary traits.

2.2.2 The defence apparatus of social wasps

The sting apparatus of the Aculeata (ants, bees, and wasps) is an anatomical and physiological derivative of the ovipositor in female Hymenoptera, able to penetrate the skin of vertebrate predators and inject pain-inducing venom (Shing and Erickson 1982). In the Hymenoptera, the apparatus is theorized to have initially evolved in solitary wasps in the role of paralyzing prey, a behaviour observable in extant solitary wasps (Schmidt 2004). However, in addition to using the sting apparatus during foraging, nonsocial hunting wasps (e.g., Pompilidae and Mutilidae) may sting vertebrate predators in self-defence, injecting highly painful venomous cocktails (Schmidt 2016). This defence mechanism helps to explain

why nonsocial wasps seem to have very few natural predators (Schmidt and Blum 1977, Schmidt 2004).

Figure 2.1. Predation on social wasps and defensive strategies to prevent colony-level predation. (A) A direct observation of an Otago skink (*Oligosoma otagense* [Squamata: Scincidae]) capturing a *Vespula germanica* (Hymenoptera: Vespidae: Vespinae) queen in New Zealand (Oceania). Photo by Ricardo Rocha Mello 2019. (B) A *Vespula vulgaris* Linnaeus nest (left) after being excavated from underground (by Jennifer M. Jandt, not included as a predation event), and (right) after peeling away the paper envelope to reveal the combs full of brood. Photos by Jennifer M. Jandt 2018. Nesting underground (out of reach) and/or building a nest envelope that blends in with the bark/stems of the tree in which it is built (crypsis) are examples of primary defensive strategies to avoid colony-level predation. (C) A *Polistes major major* (Polistinae: Polistini) foundress, on her nest, displaying a warning posture. Photo by Kevin J. Loope 2014. Visual behavioural displays are examples of secondary defensive strategies that deter nearby predators from attacking the colony.

In social Hymenoptera, stinging and biting are often used to deter potential enemies. When a colony perceives a threat, nonreproductive adult females engage and sting and/or bite potential predators, often cooperatively (Starr 1985, Nouvian, Reinhard et al. 2016). The venom injected via stinging often can translate into intense pain. Vertebrate predators associating social insect individuals or colonies with negative experiences may learn to avoid them (Schmidt 2016). This associative learning can be further reinforced by the aposematic coloration of many social bees and wasps (Vidal-Cordero, Moreno-Rueda et al. 2012).

Several lines of evidence support the effectiveness of the stinging response as an anti-predator strategy. In fact, the defensive stinging behaviour is widespread among Hymenoptera Families (e.g., Formicidae, Kugler 1979; Apidae, Nouvian, Reinhard et al. 2016; and Vespidae, Starr 1985). Also, male hymenopterans (lacking the sting apparatus) may mimic the stinging abdomen movements of females when caught by a predator

(Giannotti 2004, Schmidt 2016). Similar behaviour is displayed by many nonstinging insects that are Batesian mimics of the aposematic coloration of bees and wasps (e.g., arctiid moths [Lepidoptera: Erebidae], Simmons and Weller 2002; hoverflies [Diptera: Syrphidae], Rashed and Sherrat 2007, Penney, Hassall et al. 2014; robber flies [Diptera: Asilidae], Brower, van Zandt Brower et al. 1960).

2.2.3 Social wasps as prey

Eusocial wasp (referred to from here on simply as 'social wasp') colonies are by definition characterized by overlapping generations, a reproductive division of labour, and cooperative brood care (Wilson 1971). At the peak of its cycle, a typical social wasp colony includes a nest (the architecture of which varies across taxa), one or a few reproductives ('queens'), nonreproductive workers, and developing brood (Spradbery 1973). The brood is defenceless, and immature wasps are essentially parcels of lipid and protein, a bounty for a skulking predator (Spradbery 1973, Ying, Xiaoming et al. 2010). Nonreproductive workers protect the brood through primary or secondary colony defences. Primary defences, usually related to the choice of nesting site or nest architecture, operate before a predator initiates any prey-catching behaviour (Edmunds 1974), and decrease the chance that an encounter will take place between the colony and a potential predator. Secondary defences, such as active behavioural responses, come into play after the potential predator has encountered a nest (see below).

Generally, predators that attack social wasp nests fall into two major groups: arthropods and vertebrates. Due to their size differences and different means of finding and attacking wasps, the two pose different kinds of threats. Because social wasps have evolved virtually nonoverlapping means of defence against these predator categories, we will often distinguish arthropod versus vertebrate predation when discussing social wasp defence strategies below.

2.2.4 Primary defences: before predators arrive

Nesting out of reach

Ants are cursorial hunters that reach their prey on foot. There is probably no natural substrate, especially in the tropics, that is entirely free of scouting-and-recruiting ants, but some sites experience lower levels of predation pressure than others. Wasps that form relatively small colonies and build small nests – many *Polistes*, *Mischocyttarus*, *Leipomeles* (Hymenoptera: Vespidae), and others – on the undersides of leaf blades likely experience lower exposure to patrolling ants than do those that nest on trunks and branches (Jeanne 1979). The mass foraging army ants (*Eciton* spp. [Hymenoptera: Formicidae]) are extreme examples of ant predation in the tropics, as they cover the ground and climb vegetation in a moving mass, plundering whatever cannot escape (Chadab 1979a). However, because *Eciton* raid from the ground-up, it is possible that wasps that nest high enough in the canopy enjoy the benefit of a dilution effect. Additionally, trees and shrubs growing in water are likely completely immune from attack. The small trees, shrubs, and dead snags standing in a meter or more of water along the shores of Gatun Lake in Panama are favoured nesting sites of *Polistes canadensis* (Rau 1933). Similarly, seasonally flooded habitats in the Amazon basin are probably largely *Eciton*-free (Robert L. Jeanne and Sean O'Donnel, personal observation). *Dolichovespula maculata* (Hymenoptera: Vespidae) and *Polistes* spp. nests are particularly common in tree branches overhanging rivers in the Southeastern United States (Kevin J. Loope, personal observation). Such sites could plausibly shield nests from mammalian predators as well, and though this has not been demonstrated, these hypotheses are eminently testable in environments where social wasp colonies face well-known predation pressures, as we later discuss.

Some paper wasp species (Hymenoptera: Vespidae) frequently nest in dense vegetation and on plants sporting spines or thorns, which can be inaccessible to birds and mammals (Richards 1978). Nesting on vertical and/or relatively flat surfaces, such as tree trunks, walls, and eaves of buildings, is typical for some Neotropical Epiponini genera (*Metapolybia*, *Synoeca*, *Clypearia*, and *Nectarinella*) and sometimes observed in other genera (some species of *Polybia*, *Chartergellus*, and *Parachartergus*, as well as many

independent-founding Polistini; Jeanne 1975, Richards 1978, Edwards 1980). These sites likely reduce access to nests by climbing and flying vertebrate predators. Among the vespine wasps there are aerial and cavity/ground nesting species. Some species of *Vespa* and *Vespula* (Hymenoptera: Vespidae) use abandoned rodent holes or cavities in man-made structures or trees to initiate their nests in early spring (Akre, Greene et al. 1980, Edwards 1980, Matsuura and Yamane 1990). As the nests grow, workers gradually expand the cavities by excavating the surrounding dirt, insulation, or decayed wood (Fig. 2.1b). The only visual evidence of these nests is the traffic of foragers entering and exiting the nest, providing some camouflage against enemies, such as humans, that rely heavily on visual cues. Other vespine species build aerial nests. These can be as high as 40 m from the ground and thus inaccessible to most nonflying predators (Feás and Charles 2019).

Nesting in association with other species

Dolichoderus and *Azteca* ants, especially *Az. chartifex* (Hymenoptera: Formicidae) and allies, form huge colonies that can densely occupy entire trees and defend them and their surroundings against other ants, including *Eciton* (Delabie 1990, Somavilla, Fernandes et al. 2013). Some Neotropical paper wasps build nests regularly, and in some cases obligately, in close association with these ants. The most intimate associations involve some Epiponini wasps. Some species of *Agelaia* (Hymenoptera: Vespidae) build their nests in cavities inside the large, arboreal carton nest of *Az. chartifex* (Richards 1978). A somewhat less intimate but more common co-nester is *Polybia rejecta*, which constructs its nest within a few centimetres of the ants' nest, or even in contact with it (Servigne, Orivel et al. 2020). Wasps respond behaviourally to ant foragers near their nests. A combination of physical ejection of ant workers, wing-buzzing behaviour, and the elimination of their scent trails (see secondary defences, below) maintains an ant-free zone around the wasps' nest entrance (Servigne, Orivel et al. 2020, Barbosa, Maciel et al. 2021) and helps to offset opportunistic attacks by associated ant colonies. In drier habitats, where *Az. chartifex* is absent but *Eciton* occurs, wasps may nest in association with other ants. For example, in Quintana Roo, Mexico, and Guanacaste, Costa Rica, Epiponini wasps nest in myrmecophytic acacias occupied by *Pseudomyrmex* spp. (Espelie and Hermann 1988, Joyce 1993, Dejean, Corbara et al. 1998).

Numerous species of myrmecophyte have evolved intimate relationships with other species of ants, many of which can keep *Eciton* from their host plant. Several species of wasps in the genera *Angiopolybia*, *Pseudopolybia*, and *Mischocyttarus* nest on these plants, thereby gaining protection from army-ant predation (Herre, Windsor et al. 1986).

Nesting in trees defended by *Azteca* ants, described above, may afford social wasps some protection against vertebrates as well as army ants. Some species of host ants sting vertebrates that contact their host trees (e.g., *Pseudomyrmex* [Hymenoptera: Formicidae] species in ant acacias; Young, Kaspari et al. 1990). The relationship is mutualistic: in a study in French Guiana, none of 42 *Az. chartifex* nests with one or more *Polybia rejecta* colonies in close association were attacked by birds, whereas 9 of the 88 wasp-free nests of the ant were attacked by woodcreepers (Dendrocolaptinae) and by a woodpecker (Piciformes, Picidae; Le Guen, Corbara et al. 2015). *Polybia rejecta* is especially aggressive and at least partially effective at defending against these and other vertebrate predators. By building its nest as close to the *Azteca* nest, and often in contact with it, the wasps provide protection for the ants against these birds and possibly anteaters. Several species of independent-founding polistines also nest not just on trees with *Azteca* nests, but on myrmecophyte plants (those in close association with ant colonies) such as *Cordia* spp., *Tococa* spp., and others. These are probably commensal relationships, with the wasps gaining protection from *Eciton*, but contributing nothing to benefit the host ants (Jeanne 2020). Although nesting with ants is much rarer in Africa and Asia than in the Neotropics, the wasp *Polybioides tabidus* (Hymenoptera: Vespidae) has been reported to nest with the aggressively stinging ant *Tetramorium aculeatum* (Hymenoptera: Formicidae) in Cameroon (Dejean and Fotso 1995).

Some arboreal wasp nests are joined by passerine birds that nest nearby; birds use existing wasp nests as cues for nest-site selection and will build near artificial *Polybia rejecta* nests (Joyce 1993). Avian nesting partners could offer protection to the wasps if the birds mob approaching vertebrate predators such as raptors and monkeys (Sandoval and Wilson 2012, Barbosa, Maciel et al. 2021).

Tolerance to human presence in some species of social wasp may play a role on determining which predators target their colonies. For instance, some species of *Polistini* and *Mischocyttarini* wasps are synanthropic in the Neotropics, often nesting on man-made structures; on the other hand, most Epiponini wasps tend to rely more on natural structures

such as vegetation, and their diversity tends to be relatively reduced in highly urbanized areas (Detoni, Barbosa et al. 2018). Thus, it is possible that the predators of synanthropic social wasps are somewhat limited to other animals that also tolerate human presence. On the other hand, nesting on buildings may result in nests that are more conspicuous to predators (e.g., young *Dolichovespula* colonies are much easier for humans to notice on buildings than in trees; Kevin J, Loope, personal observation). If this increases detection and later predation by predators such as birds, then the use of buildings as a nest site could be seen as an ecological trap.

Crypsis

Adaptive nest crypsis is a response to selective pressure imposed by visually hunting predators, almost always vertebrates. Although empirical evidence of the adaptiveness of crypsis in wasp nests is lacking, paper wasp nests exhibit several putative adaptations that likely decrease their visual detection by predators by decreasing contrast from, or increasing their resemblance to, the background. In some cases, wasps may nest on substrates where their nests visually match the background. This effect can be enhanced by mosses and liverworts growing on the nest paper (Barbosa, Dias et al. 2016, Milani, Prezoto et al. 2020). Nest envelopes of the epiponine wasp *Leipomeles dorsata* Fabricius (Hymenoptera: Vespidae) constructed beneath leaf blades can closely resemble the leaf substrate, even mimicking the venation of the leaf (Richards 1978). Overall shapes of nests can evolve to lessen recognition by vertebrates, such as the stick-like nests of some Mischocyttarini and Polistini species (Vesey-Fitzgerald 1950, Richards 1978, Starr and Hook 2006, Silveira, de Souza Silva et al. 2015).

Nest crypsis could also be a strategy adopted by Vespinae. *Vespula* spp. may benefit from constructing their nests underground, but *Dolichovespula* produce large, aerial nests that often hang from trees (Akre, Greene et al. 1980). However, the nests can still be difficult to locate, at least to humans. The paper envelope can blend in with both the bark/stems of the tree it is built inside and may also be able to provide camouflage amidst vegetation or other background if hanging from trees (Feás and Charles 2019; Kevin J. Loope, personal observation).

Aposematism

An alternative strategy for both nests and groups of adult wasps is to be visually conspicuous. Well-defended nests are often placed relatively high in tree canopies, often along forest or river edges. Pale nest paper contrasts with surrounding vegetation. *Polybia striata*, *Polybia scutellaris*, *Chartergus* spp., and *Epipona niger* (Hymenoptera: Vespidae) exemplify this pattern (Jeanne 1975, Richards 1978). For groups of individuals, the sunflower-like radial array of pale-coloured adults of *Apoica pallens* (Hymenoptera: Vespidae) resting on the underside of their open nest elicits a startle response, at least in humans, when first spotted from below (Robert L. Jeanne, personal observation).

Chemical repellents

Independent-founding Polistinae (Polistini, Mischocyttarini, and Ropalidiini) and foundress queens of young colonies of Vespinae apply an ant-repelling glandular product to the nest petiole. The ant-repellent secretions are produced by specialized exocrine glands on the terminal sternite of the gaster (van der Vecht's gland; Jeanne 1970a, Post and Jeanne 1981, Keeping 1990, Kojima 1992, Martin 2017). This gland is often secondarily lost during the evolution of the Epiponini lineages that employ nonrepellent means of ant defence (see secondary defences, below; London and Jeanne 2000, Smith, O'Donnell et al. 2001). Similarly to the independent-founding Polistini, in the Vespini, at least one species (*Vespa velutina*) has been shown to utilize ant repellents on its nest during the foundation stage of the colony cycle (Martin 2017).

Sticky traps

Members of the genera *Nectarinella* and *Leipomeles* (Epiponini) erect sticky-tipped stalks around the access to their nests (Jeanne 1975, Schremmer 1977, Mateus and Noll 1997). This appears to be an effective defence against scouting-and-recruiting ants for wasp species with small colonies and with body sizes in the range of these predators, for whom active defence is less reliable.

Hardened nests

The exceptionally tough carton envelope of some arboreal-nesting swarm-founding species may be impenetrable to arthropod raiders, including chewing ants. In some species (e.g., *Chartergus artifex* [Hymenoptera: Vespidae]) the inter-comb passageways within the nest are narrowed to the size of a brood cell (Robert L. Jeanne, personal observation), possibly an adaptation allowing a single wasp to effectively block access to brood-laden combs by ants that have entered the lower chambers. The nature of the nest material can also have important effects on the capacity of the nest itself to withstand mechanical attacks by vertebrate enemies. The extremely dense and tough carton of *C. artifex*, for example, may be able to resist attacks by monkeys and birds (Robert L. Jeanne, personal observation). The transition from wood pulp (paper) to mud as a nesting material has occurred twice in the genus *Polybia*; in both mud-nesting lineages, nests are highly robust to mechanical damage, and may at least narrow the range of species that can penetrate them. *Polybia emaciata* workers often retreat into the nest upon mechanical disturbance, apparently relying on the nest as a fortress-like defence (O'Donnell and Jeanne 2002). Similarly, the hard mud nests of some stenogastrines (e.g., *Liostenogaster flavolineata* [Hymenoptera: Vespidae]) may afford them greater survival than the paper nests of their close relatives (e.g., *Parischnogaster* spp; Seirian Sumner, personal observation).

2.2.5 Secondary defences: behavioural responses to predator arrival

Physical predator removal

Due to the relevance of ants as threats to Neotropical colonies, wasps have developed anti-predator behaviours dedicated specifically to repel ant invaders from their nests. If a foraging ant makes its way onto a nest, a defending wasp may dart at it, grab it in the mandibles, and toss it from the nest or fly off with it and drop it (Chadab 1979b, Grangier and Lester 2011). If ant foraging persists, swarm-founding wasps (Epiponini) recruit nestmates to encircle the access point and repel the intruders with semi-synchronous bursts

of wing-buzzing. The mini-blasts of air are often effective in causing the ants to turn around and exit the nest (Chadab 1979b, Jeanne 1991). Following an ant invasion to their nest, wasps extensively mandibulate or lick the traversed surface, apparently expunging the ants' trail pheromone or scent (West-Eberhard 1989). Whether the behaviour removes the chemical or covers it up, possibly with the labial gland secretion used in nest construction, has not been determined. Although less well documented than the tropical examples, ground-nesting *Vespula* wasps defend their colonies from Argentine ants (*Linepithema humile* [Hymenoptera: Formicidae]) and Red Imported Fire Ants (*Solenopsis invicta* [Hymenoptera: Formicidae]) by darting at and biting ant foragers exploring the nest entrance (Kevin J. Loope, personal observation). How wasps repel the abundant subterranean foragers of these species, particularly early in colony development, is unknown.

Vibro-acoustic warnings

When facing bigger predators, namely vertebrates, adult epiponine wasps in at least three genera (*Synoeca*, *Chartergus*, and *Polybia*) respond to vibrations of the nest or its substrate by rhythmically and synchronously striking or drumming against the nest carton, thereby generating characteristic sounds that are audible to humans over distances of five meters or more (Evans and West-Eberhard 1970, Taylor and Jandt 2020, Sean O'Donnell, personal observation). Grazing livestock seem to learn to avoid patches of grass and bushes around *Po. sericea* colonies, possibly by associating their alarm sound to eventual stinging (Fábio Prezoto, personal communication). *Protonectarina sylveirae* (Hymenoptera: Vespidae) makes a characteristic high-pitched sound when attacking (Richards 1978). When their nest is disturbed, *Vespa mandarinia* workers closely approach the intruder in flight while loudly snapping their mandibles (Schmidt 2016; Robert L. Jeanne, personal observation).

Visual displays

Several species of Polistinae in the genera *Agelaia*, *Apoica*, *Brachygastra*, *Epipona*, *Polistes*, *Polybia*, *Synoeca*, as well as *Ropalidia revolutionalis* (Hymenoptera: Vespidae)

(Hook and Evans 1982), engage in visual warning displays, such as gaster-flagging, when the nest is disturbed (O'Donnell, Hunt et al. 1997; Fig. 2.1c). In response to a disturbance, workers on the nest (or on the vertebrate intruder that caused the disturbance) raise and wave the gaster, extrude the sting, and fan the wings. In some species, the gaster is conspicuously coloured, suggesting its use as a visual signal, either as a threat to predators or to communicate with nestmates. In disturbed *Polistes* spp. nests (in Malaysia), both workers and males display raised forelegs, providing a visual signal to the potential predator (Turillazzi 2003).

Chemical responses

The existence of alarm pheromones has been demonstrated in many large-colony species across different subfamilies (Maschwitz 1964, Jeanne 1981, Veith, Koeninger et al. 1984, Kojima 1994, Sledge, Dani et al. 1999, Cheng, Wen et al. 2017), as well as some small-colony species (Post, Downing et al. 1984, Bruschini, Cervo et al. 2006). These compounds are released either at the nest or when workers sting their aggressor, and serve to recruit in-nest workers to engage in defensive behaviour, as well as to attract defending workers to a specific target. Notably, experiments have failed to demonstrate venom-associated alarm pheromones in some small-colony wasps (Keeping 1995, London and Jeanne 1996), further suggesting that the selective pressures behind colony defence have been varied, and have produced a number of different strategies.

In what may be a specialized defence against small vertebrate predators, *Parachartergus colobopterus* and *Pa. fraternus* use chemicals in defence of the nest by spraying their venom in a fine mist that travels several centimetres (Jeanne and Keeping 1995, Mateus 2011). The nests are built on tree trunks and are visually cryptic, which may narrow the range of potential predators to small gleaning birds, for which an eyeful of the sticky venom may be a more effective deterrent than stinging (Jeanne and Keeping 1995).

Hiding

Contrary to popular perception of wasps being aggressive every time they are disturbed (Sumner, Law et al. 2018), some species exhibit remarkable timidity, either fleeing or hiding in response to disturbance, despite possessing a functional stinging apparatus (Hermann and Chao 1984, Strassmann, Hughes et al. 1990, O'Donnell and Jeanne 2002). Some species of *Mischocyttarus,* for example, will often hide behind their nests or, if sufficiently disturbed, fly away – avoiding a direct confrontation of any kind (Hermann and Chao 1984; Robert Jeanne and the author, personal observation). Even more drastic is the response of some Stenogastrinae wasps, which may simply 'drop' from the nest when threatened (Seirian Sumner, personal observation). While this may confuse a potential predator, or redirect their attention away from the nest, it also leaves the brood completely undefended.

Absconding

When all defensive tactics fail to repel a predator, it can be in the colony's best interest for the adults to abandon the nest and their brood, thus saving themselves for the opportunity to re-nest elsewhere. In an intriguing overlap with responses to vertebrate attacks, Neotropical swarm-founder adults readily abandon their nests when threatened by army ants (mostly *Eciton* that raid above ground; in some cases, rapid absconding can be triggered by encounters with just a few *Eciton* workers, or by their odour alone; Chadab 1979b). Observations suggest that rapid absconding may be coordinated via the wasps' alarm pheromone (Chadab 1979b).

Repeated strikes on nests by avian predators such as Red-Throated Caracaras *Ibycter americanus* (Falconiformes: Falconidae) initially induce coordinated stinging attacks, but eventually the defenders shift to rapid departure of all adults (McCann, Moeri et al. 2013). Following absconding events, surviving adults in swarm-founder colonies first cluster, then move to re-nest in a new location by following a pheromone trail (Sonnentag and Jeanne 2009).

In contrast to most polistine wasps, temperate zone *Vespula* and *Dolichovespula* colonies almost never abscond in the face of persistent or catastrophic predator attacks. This is presumably because the short seasonality of their colonies limits them from obtaining fitness gains by rebuilding a new nest following the loss of the original. Similarly, in Neotropical genera such as *Agelaia*, the scarcity and quality of nesting sites may select against absconding. While adults may leave the nest during ant raids, they return after the predators are gone, and try to rebuild the colony in the same site (O'Donnell and Jeanne 1990). Exceptionally, underground-nesting *Vespa velutina* adults may abandon nests and brood when under attack by ant predators and try to re-establish their colonies elsewhere (Xesús Féas, personal observation).

Stinging

The most notorious response to disturbance in a social wasp colony's arsenal is the painful and dangerous sting of large-bodied species such as *Vespa* and *Synoeca* (Xuan, Mai et al. 2010, de Castro e Silva et al. 2016), and the vigorous stinging attacks by tens to hundreds of workers in large-colony species like *Vespula* and *Agelaia*, in which potential predators can receive hundreds of stings (Vetter, Visscher et al. 1999). Workers are initially alarmed by movement, vibration of the nest, or by volatiles in mammalian breath (Landolt, Jeanne et al. 1998, Jandt, Detoni et al. 2020).

Despite being the most well-acknowledged active collective response to disturbance in wasps, the sting is the weapon of last resort against vertebrates. Aggressiveness in response to threats varies widely among species. The effectiveness of the sting as a defence against vertebrates is partly a function of the size of the colony and the size of the wasp. The sting of many wasp species is ineffective against a wide variety of vertebrate predators. Large numbers of epiponine colonies are taken with apparent impunity by monkeys and birds, and vespine nests are commonly attacked by large mammals and birds (see 'Results'). Yet if the defenders are aggressive enough and numerous enough, they can be effective. A study in French Guiana found that nests of *Polybia rejecta*, remarkable for its aggressiveness when defending its nests, suffered no vertebrate attacks, whereas other less aggressive epiponines did (Le Guen, Corbara et al. 2015).

If a colony is sufficiently provoked, collective or group stinging defence is coordinated, often by alarm pheromones comprised of volatiles released with venom. Similar to alarm pheromones, vibratory signals may be used to recruit wasps inside the nest to the outside in preparation for further action (Strassmann, Hughes et al. 1990, Jeanne and Keeping 1995, Taylor and Jandt 2020). The intensity and duration of collective defence by some species suggests a strong selection pressure exerted by nest predators, though we have yet to understand the links between predation frequency and type and the interspecific variation in defensive behaviours exhibited by social wasps. Colonies within a species can also vary greatly in the magnitude of the collective defensive response, which can be, but is not always, associated with colony size and developmental stage (London and Jeanne 2003, Brito, Aragão et al. 2018, Jandt, Detoni et al. 2020).

2.2.6 Who are the primary predators of social wasps?

Today, a complete spectrum of solitary to eusocial species is represented within the monophyletic lineage of Vespidae (see Fig. 4.2 in Jandt and Toth 2015), wherein eusociality evolved independently at least twice (Hines, Hunt et al. 2007, Huang, Lin et al. 2019), making it one of the best systems for studying the co-evolution of predator–prey relationships and of coordinated colony defence. To understand why social wasps have evolved these primary and secondary methods of collective defence, we first need to ask: Who are the predators of social wasps? Various predators have been sparsely identified in the literature, including other social wasps (Turillazzi 1984, Gadagkar 1991, Jeanne and Hunt 1992), ants (Bruch 1923, Jeanne 1972, Tindo, Mony et al. 2002), birds (Birkhead 1974, Huang, Lin et al. 2004, McCann, Moeri et al. 2010, van Bergen 2019), and mammals (Bigelow 1922, Perry and Manson 2008, Ying, Xiaoming et al. 2010). Do social wasps that use different primary and secondary defensive strategies receive the same predation pressure from each of these taxonomic groups; and/or is that predation pressure consistent across different regions where these wasps are biogeographically located? Moreover, by including novel data sources in our search, such as social media (Nyffeler and Vetter 2018), we may find evidence of predator–prey relationships overlooked in the literature. Here, I review the published literature and compile records from social media on the predation on vespid wasp individuals and colonies

to 1) identify the predators of social wasps; 2) explain any consistent patterns of variation in who the predators are with regards to biogeography, evolutionary and ecological traits; and 3) relate the findings above to the evolutionary history of social wasps and their predators.

2.3 MATERIAL AND METHODS

2.3.1 Literature search

We searched ISI Web of Science, Scopus, Google Books, and Google Scholar databases for publications on the predation of specific wasp taxa by systematically combining the taxonomic group (e.g., 'Wasp', 'Vespidae', or 'Polistinae') with the terms 'predator of', 'predation on', and 'attack on' (e.g., ['*Vespula*'] AND ['predator of' OR 'predation on' OR 'attack on']). The terms 'predator' and 'predation' alone were avoided due to social wasps being predators themselves, with most of the search results turning up literature focused on their ecological role as predators. Books, chapters, and reviews on social wasp biology were scanned for additional references of predation of social wasps. For each reference (from literature to other media searched), we noted: 1) the predator taxa, 2) wasp prey, 3) whether the predation event targeted an adult wasp or the colony, 4) the geographic location of the observation, 5) the medium in which the reference was found, and 6) which type of evidence did the reference constitute (defined in '2.3.3 Organization of evidence'). All reference searches (including other media below) were carried out between April and June of 2020.

2.3.2 Social media search

We used the social media platforms YouTube (youtube.com), Facebook (facebook.com), and Twitter (twitter.com) to collect additional evidence (accounts and videos) of predation events on wasp individuals and colonies. Non-naturally occurring predation events (e.g., artificial setups, such as arenas) were excluded from our data.

YouTube

Using the YouTube video database, we initially combined the search terms ‘predator of’, ‘predation on’, or ‘attack on’ with the common names for social wasps (e.g., ‘hornets’, ‘hover wasps’, ‘paper wasps’, ‘social wasps’, ‘wasps’, and ‘yellowjackets’). We then conducted a focused search using the common names for specific predator taxa (based on data from the literature search) + predation terms + common name of social wasp (e.g., ‘badger attack on social wasps’). This facilitated the database’s search mechanism, while normalizing the amount of data obtained for predators which are reported less often or lack empirical evidence in the literature (Appendix: Supplementary Table 1). For each search, we scanned the first hundred results, though the most relevant were usually shown within the first 10–20 results. That means, even though we found abundant videos of other arthropods being preyed upon, we sometimes found videos of wasps as prey being mislabelled as bees or other insects, despite never including ‘bee’ or the other descriptor as a search term.

Facebook and Twitter

We posted request for evidence of wasps being preyed upon to two Facebook ‘groups’: ‘Enthusiasts of Social Wasps’ (https://www.facebook.com/groups/414300075343425) and ‘Ecology of Vespinae’ (https://www.facebook.com/groups/869556843412485) on 17 April 2020 (Appendix: Supplementary Fig. 1A), and posted a general call on Twitter on 16 May 2020 (Appendix: Supplementary Fig. 1B). For both posts, we included an image of a *Vespula* queen on a pink Grevillea flower. This image allowed us to avoid biasing responses (e.g., an image of a bird eating a wasp might have drawn the attention from those with other examples of bird predation), while also increasing the exposure of the post, given that the image was clear and brightly coloured.

Direct e-mail to wasp researchers

We contacted wasp researchers directly or via wasp-focused listservs (Appendix: Figure 2). All responses that included unpublished observations of individual or colony-level predation were categorized as 'unpublished accounts'.

2.3.3 Organization of Evidence

Taxonomy of predators

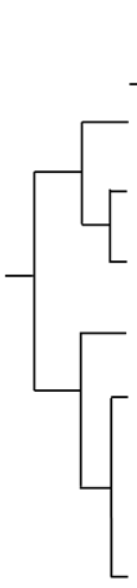
Predation records were classified as direct or indirect evidence, as well as media type (literature, social media, video, or unpublished accounts). Direct evidence is represented by both 'Empirical' studies (experiments or systematic observations where quantitative data were collected) and 'Observations' (a description, image, or video of a direct observation of a predator attacking a wasp or colony) records. 'Indirect Evidence' refers to predation claims, common lore, or instances where predation was inferred through evidence left behind following a predation event. Predators were categorized by Phylum, Class and Order, and compared across the four tribes of Polistinae, one tribe of Vespinae, and Stenogastrinae.

Social wasp subfamilies (and Polistinae tribes) were further categorized by colony size, nest architecture, colony cycle, and coordination of colony response (Fig. 2.2). We discuss patterns in predation based on these colony characteristics.

Biogeography of predators

When location of direct evidence of colony predation was provided, records were grouped by continent. We included Central America as a separate 'continent' since the social wasp fauna in the area differs greatly from the rest of North America (Hunt 2007); and used 'Oceania' instead of 'Australia' to allow the grouping of records made in western Pacific areas (combining Australia, New Zealand, and Guam). Each record was counted once for each area cited by an observer; for example, we counted D. Santoro's personal observation

Figure 2.2. Colony characteristics (colony cycle, size, nesting site, architecture, and climate) of the three social subfamilies of Vespid wasps (broken into the four tribes for Polistinae). Data are not provided for the three solitary subfamilies (Euparagiinae, Masarinae, or Eumeninae), as they were not included in the data presented in this study. The primary colony predator taxon is based on data shown in Table 2.2.



Subfamily	Tribe	Colony Cycle	Colony Size	Nesting Site	Nest Architecture	Climate	Primary Colony Predator Taxon
Stenogastrinae		Perennial	2-10	Under eaves, leaves, or vegetation	Single-layer comb, no envelope	Tropical	Hymenoptera
Euparagiinae		Solitary					
Masarinae		Solitary					
Eumeninae		Solitary					
Vespinae	Vespini	Seasonal	10^2-10^6	Underground (cavity) or aerial	Paper envelope	Tropical + Temperate	Carnivora
Polistinae	Epiponini	Perennial	10^2-10^7	Vegetation, cavities, under eaves or leaves	Paper or mud envelope OR single-layer comb, no envelope OR multi-layer comb, no envelope	Tropical	Hymenoptera
	Mischocyttarini	Seasonal/ Aseasonal	10-10^2	Under eaves or leaves, cavities	Single-layer comb, no envelope	Tropical/ Subtropical	Hymenoptera
	Polistini	Seasonal	10-10^2	Under eaves or leaves	Single-layer comb, no envelope	Tropical + Temperate	Hymenoptera
	Ropalidiini	Seasonal/ Indeterminate	10-10^3	Vegetation, under eaves or leaves	Single-layer comb, no envelope OR paper envelope	Tropical/ Subtropical	Hymenoptera

on the predation of humans on *Vespula* in Japan and China as two distinct observations in Asia.

Evolution of predators

Using the results from the literature and social media searches, we mapped the evolutionary origins of vertebrate predators with the most records of predation attempts of social wasp colonies: three Classes (Aves, Mammalia, and Reptilia), and the top two Orders in the Classes Aves (Accipitriformes and Passeriformes) and Mammalia (Carnivora and Primates). Among invertebrate predators, we mapped the evolutionary origins of hymenopteran Family Formicidae (ants) and Genus *Vespa* (hornets, Family: Vespidae). Along this evolutionary map, we also plotted the evolutionary origins of Aculeata (stinging wasp, bee, and ant ancestor), Vespidae, the common ancestor of Polistinae and Vespinae (the hypothesized evolution of eusociality in those groups), and the origins of the three social wasp subfamilies in Vespidae (Vespinae, Polistinae, and Stenogastrinae).

2.4 RESULTS AND DISCUSSION

2.4.1 Search Results

Sampling efforts yielded a total of 720 records of direct evidence ('Observation' and 'Empirical Studies' data; n=489, 67.9%) and 'Indirect Evidence' (n=231, 32.1%) of predation events on social wasps (summarized in Table 2.1; Appendix: Supplementary Table 2). Most events were obtained from the literature search (n=509, 70.7%), followed by YouTube video data (n=119, 16.5%), e-mail correspondence (n=72, 10.0%), and Facebook + Twitter (n=20, 2.8%). Although most of the data were published in scientific literature, our diverse methods of data collection increased our sample size and allowed the inclusion of previously unpublished predator observations (e.g., predation of Ropalidiini wasps by macaques; Anindita Brahma, personal communication), and surprising new observations (e.g., attack on a stenogastrine wasp by a predatory nematode; Stefano Turillazzi, personal communication).

Although empirical studies were the least common reference type (n=61, 8.5%), all were published in the scientific literature (Table 2.1). 'Observations' (n=428, 59.4%) and 'Indirect evidence' (n=231, 32.1%) dominated the reference types reported. It must be noted that combining a literature review and data collection in social media with search and communication being predominantly done in the English language undoubtedly leads to a strong bias in the dataset (here represented by the inflated number of records in the North American continent). Besides language barriers, socio-economic aspects such as access to the internet and overall level of education in a population can significantly impair researchers' abilities to draw conclusive global trends when conducting reviews (Doherty, Filion et al. 2021, Poulin, Bennett et al. 2021). Still, when the significant gap in literature regarding worldwide trends in social predators is considered, these biases must be put in perspective; careful interpretation of results can be used as inspiration for future investigations with stronger empirical foundations.

Table 2.1. Summary of records collected on individual- and colony-level wasp predation (combined). Direct Evidence is split into empirical studies and direct observations of predation events. Indirect evidence includes common lore or inferred predator based on post-predation damage. Values represent total number of references for each data collection type (literature search, social media campaign, video, and unpublished observations).

	Evidence Type	Literature	Social Media		
		Published	Facebook + Twitter	YouTube	E-mail correspondence
Direct evidence	**Empirical Study** Experiments or systematic observations where quantitative data were collected	61	0	0	0
	Observation A description of a one-off or multiple predation events that were observed first-hand	229	18	114	67
Indirect evidence	Common lore regarding predation, or a suggestion of a predator event based on post-predation damage	219	2	5	5

Predation on wasp colonies was recorded most often in Polistinae (52.7%), whereas predation on individual wasps was recorded most often in Vespinae (42.0%). This is likely

due to the fact that Polistinae nests are often exposed (Fig. 2.1C) when compared with Vespinae, which often build nests in cavities or underground (Spradbery 1973).

2.4.2 Taxonomy of predators

Predator type varied across the subfamilies within Vespidae, and by whether they preyed upon individuals or colonies (Table 2.2). Direct evidence on individual polistine and vespine wasps shows they were primarily preyed upon by arthropods (Classes: Araneae and Insecta) and birds (Class: Aves). Amphibians, carnivores, and reptiles were also observed eating individual wasps. Only one observation of predation on a Stenogastrinae individual was observed, and it was particularly unusual. Stefano Turillazzi reported an observation of a predatory nematode consuming (not parasitizing) a stenogastrine wasp.

At the colony level, direct evidence of predation on Polistinae consists mostly of records of hymenopteran predators (Fig. 2.3); ants were most often observed preying upon polistine wasps (Jeanne 1972, Strassmann 1981, O'Donnell and Jeanne 1990, Barbosa, Maciel et al. 2021), followed by *Vespa* hornets (Matsuura and Sakagami 1973, Matsuura 1991). *Vespa* will attack and collect adults as well as brood as prey items to bring back to the colony; *Vespa mandarinia* can recruit nestmates to join them in the attack on the colony (Ono, Igarashi et al. 1995). Passeriformes (e.g., crows) had the second highest record of colony predation (Raw 1997). Despite collecting the most direct evidence of Hymenoptera as predators, indirect evidence of Hymenoptera as predators of polistine wasps in the literature were over-represented, whereas Passeriformes predators were never identified (indirectly) as predators of polistine colonies (Fig. 2.3).

Carnivores were the most common predators of colonies of Vespinae, followed by Accipitriformes (eagles, hawks, and kites; Fig. 2.3). The highly specialized *Pernis* honey buzzard can individually raid vespine colonies for brood combs, seemingly unaffected by the wasps' aggressive response (Gamauf 1999, Huang, Lin et al. 2004). Among the Carnivore predators, we found the most direct evidence of badgers and weasels (Family: Mustelidae; Blackith 1958, Lanszki and Heltai 2007) preying upon vespine wasps, followed by bears (Family: Ursidae; Mealey 1980). These carnivores possess strong paws with long claws, effective in digging up and tearing apart the wasp nest envelope (in the case of *Vespula*),

undoubtedly playing a role in the success of carnivores as colony predators. Indirect evidence of Carnivora predators in the literature were slightly overrepresented; whereas indirect evidence of Accipitriformes predation were slightly underrepresented relative to the direct evidence found (Fig. 2.3).

There were only nine records of direct evidence of predation on stenogastrine colonies (Table 2.2). Among these, *Vespa* hornets were identified as the most common predator (n=6), followed by ants (n=2) and one record of a reptile consuming a stenogastrine colony. Additionally, Hymenoptera were also the only taxonomic group reported in indirect evidence as predators on stenogastrine colonies.

We compared traits characteristic of the three subfamilies of Vespidae, and four tribes of Polistinae (colony cycle, colony size, nest building patterns, and climate) with the primary colony predator taxon (Fig. 2.2). Social wasps varied in terms of colony cycle (seasonal and perennial), colony size (2–107 adults produced in a colony), and the types of nest that were constructed. Most subfamilies and tribes were primarily preyed upon by Hymenoptera, and this seemed most closely linked to the fact that those groups were predominantly located in tropical climates. Vespinae tend to be found in temperate climates, and they were preyed upon mostly by Carnivora. Polistini are found in both temperate and tropical climates, yet epiponines (exclusively tropical) had more direct evidence of predation by mammals among the Polistinae tribes.

2.4.3 How does predation pressure across these taxonomic groups vary geographically?

We found direct evidence of predation on social wasp colonies in every inhabited continent (all except Antarctica; Fig. 2.4). Predation on polistine colonies was recorded in every continent, whereas predation on vespine colonies was only recorded in North America, Europe, and Asia. Stenogastrine wasps are only found in Asia, and that is where its predation events were recorded (Turillazzi 1991).

Table 2.2. Summary of direct evidence of **(A)** individual-level and **(B)** colony-level predation on the three social subfamilies of Vespidae (Polistinae, Stenogastrinae, and Vespinae). Direct evidence includes instances when predation was directly observed and reported in the literature, social media, or other sources. Columns listed as "Unknown" represent wasp prey that could not be confirmed from video or was described vaguely in text as "social wasp" or "Vespid wasp". "Paper wasps" were categorized as "Polistini", as that is the accepted common name for *Polistes* wasps, and "Yellowjackets" and "Hornets" were categorized as "Vespini", as those are common names for *Vespa*, *Vespula* or *Dolichovespula*. Data are presented for predator Phylum (grey rows), which are broken into Class and most commonly observed Orders (white rows).

	POLISTINAE					STENOGASTRINAE	VESPINAE	UNKNOWN	
A. Individual Predation	**Epiponini**	**Mischocyttarini**	**Polistini**	**Ropalidiini**	**Unknown**		**Vespini**		**TOTAL**
ARTHROPODA	5	4	30	0	2	0	59	2	102
ARACHNIDA	1	4	14	0	1	0	16	1	37
INSECTA - Hymenoptera	0	0	5	0	0	0	18	0	26
INSECTA - Other	4	0	8	0	1	0	25	1	39
CHORDATA	21	1	9	0	1	0	37	45	114
AMPHIBIA	0	0	1	0	0	0	3	0	4
AVES – Accipitriformes	1	0	1	0	0	0	3	1	6
AVES – Passeriformes	10	0	1	0	0	0	10	27	48
AVES - Other	10	1	4	0	1	0	6	16	38
MAMMALIA – Carnivora	0	0	1	0	0	0	11	1	13
MAMMALIA – Primates	0	0	0	0	0	0	0	0	0
MAMMALIA - Other	0	0	0	0	0	0	0	0	0
REPTILIA	0	0	1	0	0	0	4	0	5
NEMATODA	0	0	0	0	0	1	0	0	1
TOTAL Individual Predation	**26**	**5**	**39**	**0**	**3**	**1**	**96**	**47**	**217**

Table 2.2 (cont.)

	POLISTINAE					STENOGASTRINAE	VESPINAE	UNKNOWN	
B. Colony Predation	**Epiponini**	**Mischocyttarini**	**Polistini**	**Ropalidiini**	**Unknown**		**Vespini**		**TOTAL**
ARTHROPODA	23	7	28	14	5	8	10	6	101
ARACHNIDA	0	0	1	0	0	0	0	0	1
INSECTA - Hymenoptera	23	7	22	13	5	8	10	6	94
INSECTA - Other	0	0	5	1	0	0	0	0	6
CHORDATA	30	9	31	4	3	1	71	22	171
AMPHIBIA	0	0	0	0	0	0	0	0	0
AVES – Accipitriformes	4	0	5	1	0	0	19	8	37
AVES – Passeriformes	10	2	13	1	1	0	4	6	37
AVES – Other	11	6	3	1	0	0	3	4	28
MAMMALIA – Carnivora	0	0	4	0	0	0	29	3	36
MAMMALIA – Primates	3	1	4	1	1	0	11	1	22
MAMMALIA – Other	2	0	0	0	0	0	5	0	7
REPTILIA	0	0	2	0	1	1	0	0	4
NEMATODA	0	0	0	0	0	0	0	0	0
TOTAL Colony Predation	**53**	**16**	**59**	**18**	**8**	**9**	**81**	**28**	**272**

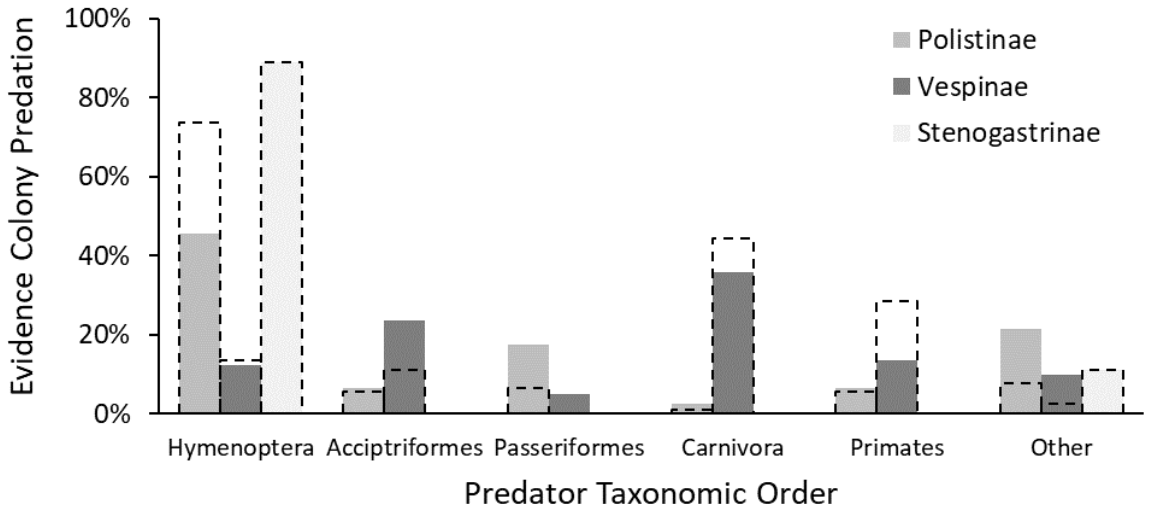


Figure 2.3. Direct versus indirect evidence of predation on social wasp colonies by the top five predators (see Table 2.2), and all other predators combined ('Other'). Percentage of direct evidence of predation is shown with filled bars (gray = predation on polistine colonies, dark gray = predation on vespine colonies, light gray = predation on stenogastrine colonies); and percentage of indirect evidence is shown with dashed bars. There was no indirect evidence of Passeriformes predation on vespine colonies. Percentages are calculated based on the total evidence (direct or indirect percentages calculated separately) for each subfamily.

Evidence of colony predation in Africa and Oceania was limited to 6 and 5 records respectively, compared with >30 records for each of the remaining continents. This small amount of evidence suggests our findings probably do not represent the full picture of prey–predator relationships in those regions. For instance, despite the presence of Vespinae wasp populations in Africa and Oceania (Lester 2018), along with predator clades that are widely reported to prey on wasps elsewhere in our data (e.g., *Pernis apivorus* [Accipitriformes: Accipitridae] honey buzzards in Africa, Bijlsma 2002); weasels in Oceania, King 2017), we could find no evidence of these predation events occurring in such areas. The same bias can be observed by separating records by countries, which also varied in terms of predation records. For example, Brazil, Costa Rica, French Guiana, and the United States each had at least 15 records, whereas the remaining countries had few or none.

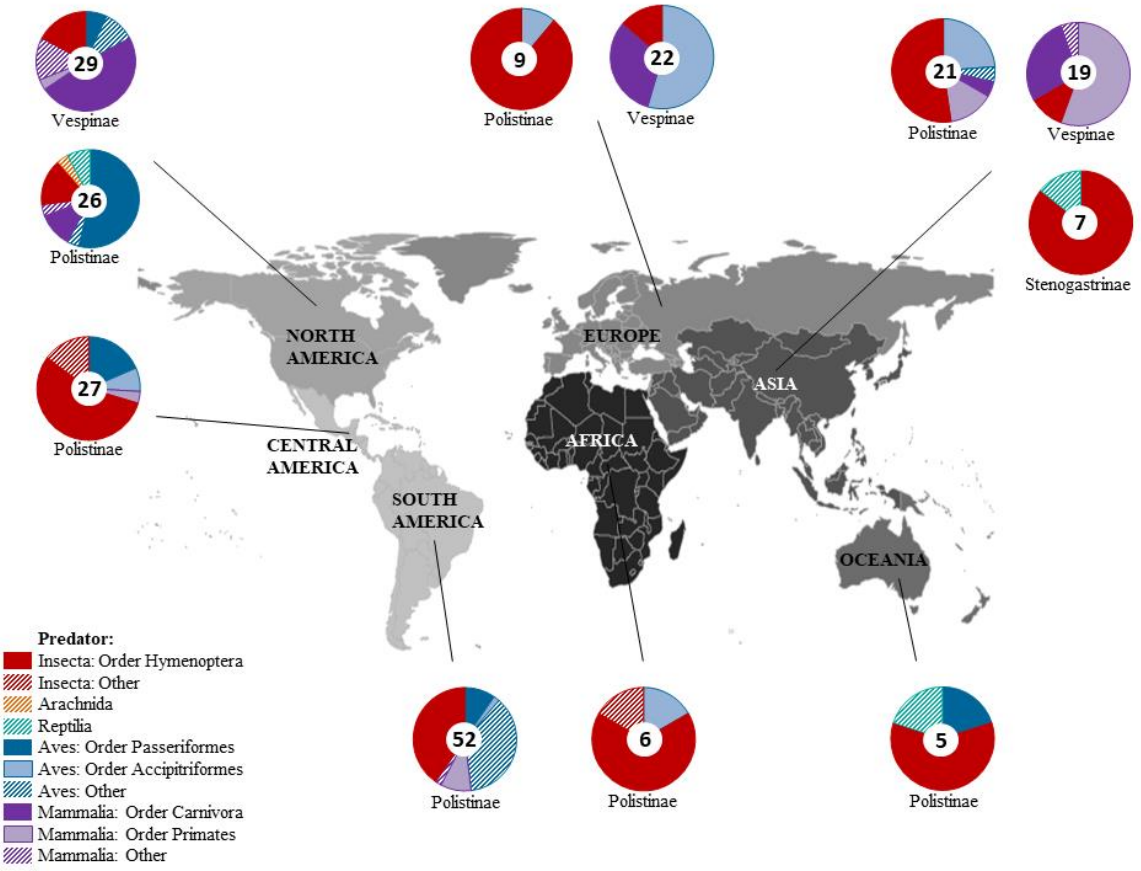


Figure 2.4. Relative frequency of direct evidence of predation events on colonies of the three social subfamilies of vespid wasps across the six continents where Vespidae are found. A separate pie chart was made for each subfamily in each of the continents where colony predation was recorded and the predator was defined. Pie charts are linked to the continent where its data were recorded by black lines. The most common Orders of predator are shown with solid colours, remaining Orders are combined and represented by 'Class: Other' as striped colours. A grey gradient was used to differentiate continents with shared borders. Continent map was modified from original template generated in R (version 4.0.3, R Core Team 2020) using the package 'maps' (version 3.3.0, Becker and Wilks 1993).

Africa

In the African continent, ants were the primary predators of polistine colonies. Of note, we also uncovered a record of predation of *Belonogaster petiolata* (Hymenoptera: Vespidae: Polistinae, Ropalidini) by *Hoplostomus fulgineus* (Coleoptera: Scarabicidae). Although there are Vespinae in Africa (Tribe and Richardson 1994), we found no direct evidence of colony predation events.

Central America

In Central America, ants and passeriform birds were the main predators of polistine colonies. *Eciton* army ants comprised two-thirds (67%) of the hymenopteran examples. Army-ant colonies can raid entire wasp colonies without facing significant opposition. Birds are also reported predators of Central American Polistinae, though with fewer direct observations. Records of predation by non-Accipitriformes, non-Passeriformes birds (Ibycter americanus), and Passeriformes (especially in the Family Corvidae) on Epiponini colonies were common. Central America also had the most predation on wasp colonies by nonhuman Primates, all in the Family Cebidae (capuchin monkeys).

North America

In North America, Passeriformes were the most common predator of polistine colonies. Still, the taxonomic diversity of polistine predators in North America was the highest for all continents (which may be linked to the higher number of predation events recorded in this region). Along with other insects (including ants) and various chordates, we also found direct evidence of a black widow spider (*Latrodectus mactans* [Araneae: Theridiidae]) preying upon a colony of *Polistes apachus* (Hymenoptera: Vespidae) (Gibo and Metcalf 1978). Carnivora, namely bears (Family: Ursidae), skunks (Family: Mephitidae), and badgers (Family: Mustelidae), were the primary predators of vespine colonies in North America.

South America

In South America, ants were the primary predators of polistine colonies, although non-passeriform/non-accipitriform birds were also commonly recorded. Bird predation in South America was mostly recorded for the Red-Throated Caracara (*Ibycter americanus*) upon Epiponini, Polistini, and Mischocittarini (McCann, Moeri et al. 2010, McCann, Moeri et al. 2013, McCann, Moeri et al. 2015). In South America, we found the only record of

predation by bats, *Phylloderma stenops* (Chiroptera: Phyllostomidae), upon *Polybia sericea* (Jeanne 1970b), and by bees, *Trigona hypogea* (Hymenoptera: Apidae), upon *Agelaia flavissima* (Hymenoptera: Vespidae) and *Polybia emaciata* (Mateus and Noll 2004). Among the ant predators, *Eciton*, *Camponotus*, and *Crematogaster* were common predators of wasp colonies in South America. Like Africa, although vespine populations have established in the temperate regions of Chile and Argentina (Masciocchi and Corley 2013), we did not find evidence of predation on vespine colonies in South America.

Asia

Asia is the only continent where all three social wasp subfamilies are present, and where predation on each was recorded. Ants are common predators of polistine colonies in Asia, but here, two of the most seemingly specialized groups of social wasp predators emerged: *Vespa* hornets and *Pernis* honey buzzards. Predation by *Vespa tropica* is one of the foremost causes of colony failure in *Polistes chinensis* Fabricius in Japan, and hornets may play a significant role in controlling Asian polistine populations (Miyano 1980). Although I only found seven records of direct evidence for stenogastrine colony predation, *V. tropica* was the primary predator of these colonies. Still, it is worth noting that an unidentified gecko (Reptilia: Gekkota) was recorded preying upon a colony of *Parischnogaster mellyi*. It is likely that predators of Stenogastrinae are largely unreported in the literature; however, predation on stenogastrine colonies has been described as remarkably rare in nature (S. Turillazzi, personal communication). Finally, vespines were almost only recorded being preyed upon by mammals (Primates, all Hominidae; and Carnivora), with a smaller proportion of hymenopteran predators recorded (including *Vespa*). Notably, human consumption of wasps was the primary cause of vespine predation in Asia, showing a unique prey–predator dynamic as well as an important cultural relationship between human societies and social wasps compared with other continents (Nonaka 2010, see '*Humans as predators across continents*' below).

Europe

In Europe, similarly to Asia, ants, *Vespa* hornets, and the European honey buzzard (i.e., 'specialized predators') were the primary predators of polistine wasp colonies. Honey buzzards were also a major predator of Vespinae. The European badger *Meles meles* (Carnivora: Mustelidae) was the most common carnivore predator on European vespines, consistent with the carnivore predation data in North American on vespine colonies.

Oceania

In Oceania, ants were the primary predators of polistine nests, and Ropalidini colonies were the only tribe to be repeatedly recorded with direct evidence of predation. As in Asia and North America, Oceania had one record of geckos attacking Polistinae colonies (Davide Santoro, personal observation). Similarly to South America and Africa, despite vespine wasp colonies having established in this region, we found no evidence of colony-level predation.

Humans as predators across continents

Human predation on social wasps was recorded in North America and Asia (not including nest removal for the purpose of population control or other nonconsumption-related purposes). Insects are recognized as an important food source for early hominids (Arnold 2017). In fact, contemporary consumption of edible insects by humans is still common among one-third of the world's population (Raheem, Carracosa et al. 2019). In the specific case of social wasps, researchers have documented long traditions of harvesting wild nests to eat larvae and pupae. Moreover, collectors have also developed practices that can be understood to some extent as domestication, such as the rearing and keeping of wasps in human-made enclosures for their entire life cycle (Payne and Evans 2017, Saga 2019). Wasp brood is highly nutritious also for humans, being rich in proteins and containing essential amino acids (Ying, Xiaoming et al. 2010). Wasps are notably appreciated as food in parts of Asia, being commercially available at high prices depending upon their species (Nonaka 2010). Another aspect of human exploitation of social wasp products can be seen in the use of nests in

medicine recipes since ancient times (Chinese Pharmacopoeia Commission 2010). Referred to as *Nidus Vespae*, contemporary science has investigated the therapeutic use of nests for human health, suggesting medical significance for the treatment of rheumatoid and psoriatic arthritis, dental disease, respiratory disorders, cervical erosion, and other disorders (Wang, Zhang et al. 2013).

2.4.4 The co-evolution of predators and their social wasp prey

Stinging aculeate hymenopterans evolved around 200 million years ago (mya; Peters, Krogmann et al. 2017, Huang, Carpenter et al. 2019, Tang, Zhu et al. 2019). These solitary stinging ancestors to social insects likely used their stings to immobilize prey before carrying it back to the nest to feed their larvae. Vespidae evolved around 166 mya, which is also when stenogastrines split from the rest of the Vespid wasps (Huang, Carpenter et al. 2019). There were two separate origins of eusociality in Vespidae (Hines, Hunt et al. 2007, Huang, Carpenter et al. 2019), once in the common ancestor of Vespinae and Polistinae (approx. 75–80 mya; Huang, Carpenter et al. 2019, Tang, Zhu et al. 2019) and once in Stenogastrinae (between 166 and 29 mya; Huang, Carpenter et al. 2019). Vespinae and Polistinae originated ~62 and 55 mya, respectively (Huang, Carpenter et al. 2019).

Among the five main predators of social wasps, the ants (Family: Formicidae) originated before either origin of eusocial evolution in Vespidae (135–115 mya; Brady et al. 2006, Huang, Carpenter et al. 2019). Primates (74 mya) and Carnivora (63 mya; Class: Mammalia) originated before the three social subfamilies radiated (Fig. 2.5; Springer et al. 2003) and Passeriformes (47 mya) and Accipitriformes (44 mya; Class: Aves) originated after Vespinae and Polistinae (Nagy and Tökölyi 2014, Oliveros, Field et al. 2019). In other words, the coordinated response to low levels of carbon dioxide or vibrations—both indicators of a vertebrate predator—likely evolved before these extant predator lineages did.

Coordinated defensive behaviour toward vertebrates may have evolved in the early social vespid ancestor in response to predation pressure from small mammals (225 mya; Kemp 2005), birds (116 mya; Lee, Cau et al. 2014), and possibly lizards (>300 mya; Laurin and Reisz 1995) and other reptiles, many of which may be extinct today. This predation pressure may have driven the evolution of the response of 10s or 100s of individuals to leave

the nest and attack an intruding predator, and the prevalence of defensive strategies with varied degrees of aggressiveness employed throughout Vespidae, and not limited to one subfamily or tribe. Those wasps that responded fast enough and with strong enough venom would have succeeded in deterring the potential predator.

Individual stinging insects can be consumed by a variety of predators (e.g., Table 2.2). Some of these predators have evolved strategies to avoid the harmful effects of toxic venoms. Although they were not found to be a common colony predator, reptiles were observed to prey upon individuals and/or colonies of all three subfamilies of social vespids. Horned lizards have evolved an innate resistance to harvester ant venom, one of the most toxic venoms to mammals, through a combination of a factor in their plasma (Schmidt, Sherbrooke et al. 1989), and a slippery and viscous mucus that lines their mouth and digestive system (Schmidt 2016). In spite of being able to avoid the painful or lethal effects of venom, this resistance does not protect the lizard from a colony response (Schmidt 2016). In other words, in the co-evolutionary arms' race of predator and prey, small predators may have evolved tolerance to an attack from a single stinging insect, but the evolutionary response of coordinated defensive attack (e.g., harvester ant attack en masse by biting) means that small vertebrate predators could be quickly overwhelmed by their potential prey.

Among the predators identified here, two orders of birds (Passeriformes and Accipitriformes) and two orders of mammals (Primates and Carnivora) originated after eusociality evolved in polistine and vespine wasps (Fig. 2.5). These large, robust creatures would have stumbled across a social wasp nest, filled to the brim with proteinaceous larvae, and tried their luck at having a treat. For instance, the observations of hawks attacking vespid colonies suggests that the birds are somewhat aware of the wasps' retaliation, but overall unfazed and undeterred (van Bergen 2019, video 39; Appendix: Supplementary Table 3). Similar observations of carnivore predators also show that these mammals can withstand multiple stings to obtain their prize (video 33; Appendix: Supplementary Table 3). Social wasps' defensive response may have evolved alongside predation: as colonies became larger, they attracted more, larger and more sting-tolerant predators, and experienced greater predation pressure. This, in turn, may have selected for stronger defensive responses by wasp colonies, which had greater numbers of workers to allocate toward defence. This positive feedback may explain the wide variation in defensive response across species: while most

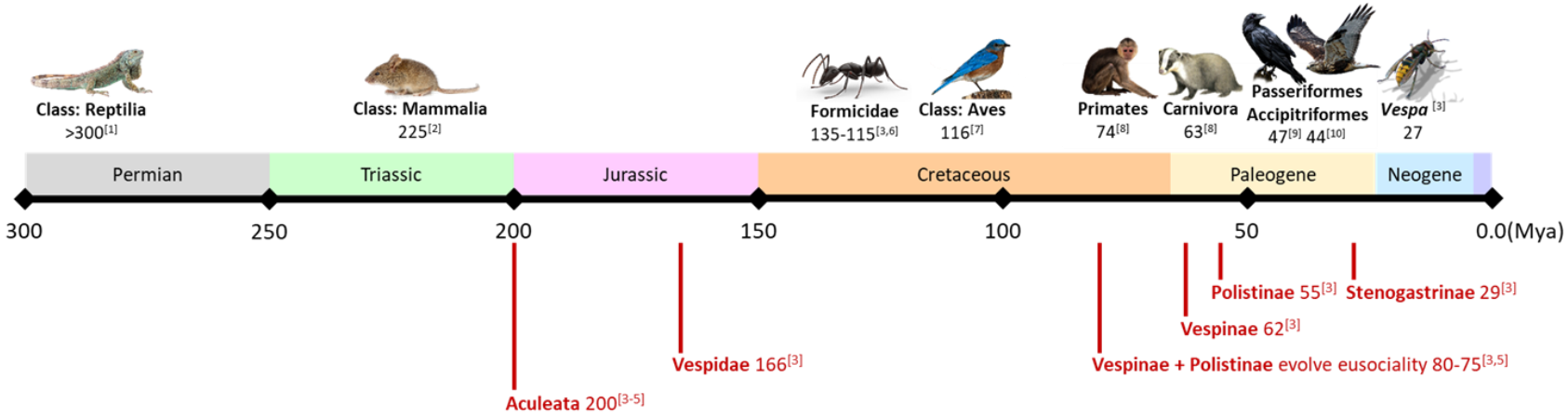


Figure 2.5. Evolutionary origins of predators and prey. (Top) The evolutionary origins of the top three Classes of Chordates (Reptilia, Mammalia, and Aves), and the top four Orders (from Mammalia and Aves) of colony predators. Ants and *Vespa* hornets are shown separately to represent evolutionary origins of the top hymenopteran predators. (Bottom) Evolutionary origins of Aculeata, Vespidae, and the three main subfamilies of Vespidae (Polistinae, Stenogastrinae, and Vespinae). It is hypothesized that eusociality evolved twice in the Vespidae Family, once in Stenogastrinae (sometime between 166 and 29 mya) and once in the common ancestor of Vespinae + Polistinae (approximately 80–75 mya). **References**: [1] (Laurin and Reisz 1995); [2] (Kemp 2005); [3] (Huang et al. 2019); [4] (Peters et al. 2017); [5] (Tang et al. 2019); [6] (Brady et al. 2006); [7] (Lee et al. 2014); [8] (Springer et al. 2003); [9] (Oliveros et al. 2019); [10] (Nagy and Tökölyi 2014).

social wasps can sting in response to a threat, many species, particularly with small colonies, often flee rather than defend (*Mischocyttarus* spp., *Metapolybia* spp.; Hermann and Chao 1984; Kevin J. Loope, personal observation), or mount a relatively weak defence (*Vespula consobrina*; Akre, Reed et al. 1982, Gaul 1952). However, their close relatives produce impressive defensive responses (e.g., *Vespula germanica*; Jandt, Detoni et al. 2020, *Synoeca*; de Castro e Silva, Oliveira et al. 2016). The *Vespa* colonies, with their large workers, strong venom, and terrifying aggressiveness may be the ultimate evolutionary response to attacks on vespine colonies.

2.5 CONCLUSION

Wasp colonies are preyed upon by a diverse variety of natural enemies. Social wasps have evolved various primary (nest construction) and secondary (coordinated behaviour) responses to defend the colony and avoid predation. The most common predators of social wasps colonies tend to be other Hymenoptera (specifically ants and hornets), carnivores (Order: Carnivora), primates (Order: Primates), perching birds (Order: Passeriformes), and hawks (Order: Accipitriformes). The prevalence of these different predator groups varied across different geographical regions, and we highlight gaps in some regions where colony predation has not been recorded, despite the presence of social wasps. The specialized behaviour of some predators (e.g., honey buzzards) may be a good starting point to deepen our understanding of the co-evolutionary relationships between predators and social wasp prey.

Chapter 3

Older, But Not Wiser: Social Wasp Colony Defensive Behaviour Decreases with Time, Not Experience[3]

3.1 ABSTRACT

Behavioural shifts in animals are often driven by ontogenetic processes such as ageing or life experiences, through changes in their physiology and/or gene expression. Colonies of eusocial animals such as social hymenopterans may experience behavioural shifts at the colony-level due to maturation and/or experience throughout the colony cycle. In this study, we investigated the nest defence behaviour of social wasp *Vespula vulgaris* colonies and whether behavioural changes over time or differences in experience with simulated threats can affect aggressive response. Colonies were divided into two treatment groups and experimentally disturbed by simulating a mammal attack. This procedure was carried out for "hi-freq" colonies on a weekly basis, whereas "lo-freq" groups were disturbed once every three weeks. We show that nest defence behaviour in *V. vulgaris* decreases over time, towards the end of the colony cycle, regardless of how frequently they faced a simulated predator attack. We found no evidence that undisturbed foraging activity or nest size could be used to predict defensive behaviour. Our results show anti-predator colony behaviours in *Vespula* are influenced by the colony cycle, possibly through demographic shifts over time: colonies near their decline are less likely to respond to a simulated threat when compared to earlier in the season. We propose colony behaviour is strongly engrained in seasonality and internal development, to a degree that it might overrule the effect of interactions with potential predators. We further discuss the potential intrinsic factors that may lead to this shift in behaviour as the colony matures.

Key words: Aggression; habituation; nest defence; ontogeny; Vespidae; *Vespula*.

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3.2 INTRODUCTION

There are ecological and evolutionary implications when animal populations maintain consistent variation in behavioural traits across time or contexts (behavioural types; Sih, Bell et al. 2004a; Bengston, 2018; Jandt, Bengston et al. 2014). Inherited behavioural traits play an important role in the persistence of behavioural variation and types, within populations over evolutionary time (Bengston, Dahan et al. 2018). However, an individual's experiences and developmental stages may also influence behaviour throughout a lifetime (Biro and Stamps 2008; Bengston and Jandt 2014). Physiological changes that occur as an organism develops and grows (e.g. changes in hormonal levels and/or metamorphosis) may drive shifts in behavioural consistency, especially during the transition between stages of the life cycle, such as reaching sexual maturity (Bell and Stamps 2004; Stamps and Groothuis 2010; Wilson and Krause 2012). Furthermore, the environmental variability that an individual experiences throughout development, such as climate, predation risk, or food availability, can influence behavioural type (Sih, Bell et al. 2004b; Bell and Sih 2007; Pinter-Wollman, Gordon et al. 2012; Stahlschmidt, O'Leary et al. 2014; Urszán, Garamszegi et al. 2015).

From a developmental perspective, ageing can significantly influence behavioural shifts throughout an individual's lifespan (Biro and Stamps 2008; Stamps and Groothuis 2010; Bengston 2018). For example, in the field cricket (*Gryllus integer*), females remain consistently bold as they metamorphose from juvenile to adult, while males tend to become shy, possibly due to costs associated with reproductive behaviour (Hendrick and Kortet 2012). In some bird species, aggression-linked behavioural types present in nestlings may shift or disappear in adults (aggression towards handlers in blue tits, *Cyanistes caeruleus*, Class and Brommer 2015; aggression towards rivals in zebra finches, *Taeniopygia guttata castanotis*, Wuerz and Krüger 2015). Inconsistency of behavioural types through development can also be seen in zebrafish reared in laboratory conditions – risk-taking behaviours are consistent within the larval or the adult stage, but not between them (Alfonso, Peyrafort et al. 2020).

Experience may also influence behaviour over time, particularly experiences with predation risk. For instance, in a Neotropical freshwater fish (*Poecilia vivipara*), behaviours

associated with boldness, activity, and sociability may arise in response to predation risk, and individual variation in boldness is maintained even if predators are removed (Sommer-Trembo, Petry et al. 2017). In a similar fashion, Florida scrub-jays (*Aphelocoma coerulescens*) tend to display increased neophobic behaviours towards novel objects in their environments after recent stress-inducing experiences (experimental capture and restraint; Bebus, Jones et al. 2020). In laboratory conditions, mud crabs (*Panopeus herbstii*) that are conditioned by exposure to non-lethal predator cues show subsequent reduction in their activity levels, presumably as a defence behaviour for increasing survival rates (Toscano 2020).

Experience with predation-events can also modulate behaviour through habituation or sensitization. An animal may become habituated to a stimulus (e.g., decrease its aggressive response) if repeated exposure does not result in significant benefits or costs (Thomson and Spencer 1966). Habituation is most often observed in studies where fake predator models are presented to organisms to gauge reaction. If there is no actual threat involved, the animal will gradually learn to ignore the model and decrease its defensive reaction (Raderschall, Magrath et al. 2011; Pelliteri-Rosa, Bellati et al. 2017; DeRango, Schwartz et al. 2019). Conversely, repeated exposure to a stimulus may lead to the animal becoming sensitized to the stimulus, reacting faster and more intensely each time it is exposed (Kandel and Schwartz 1982). Sensitization may allow animals to recognize cues that relate to a predator and employ defence mechanisms more quickly and/or effectively (Mason, Watkins et al. 2014; Sommer-Trembo, Petry et al. 2017; Toscano 2020).

In contrast to the examples listed above, social insects, such as ants, bees, and wasps, are unique in that behaviour can be studied at multiple levels of organization (Szathmáry and Maynard Smith 1995). That is, individuals within a colony, and colonies within a population, can maintain distinct behavioural types across time and/or context (Jandt, Bengston et al. 2014; Wright, Lichtenstein et al. 2019). For example, at the individual level, physiological changes among workers as they age, such as hormone shifts linked with temporal polyethism (age-determined division of labour), may correspond to an increase in aggression and activity (i.e. ants: Vieira, Fernandes et al. 2010; bees: Seeley 1982; Pearce, Huang et al. 2001; wasps: Hurd, Jeanne et al. 2007, Santoro, Hartley et al. 2015). At the colony level, growth (increasing worker numbers) and development (shifts in brood:worker ratio, development of

reproductives) may also correspond with shifts in behavioural phenotypes. Social insect colonies are most aggressive during the growth phase (when investment in brood - larvae and pupae - is maximized) compared to the juvenile or mature phase of colony development (ants: Gordon 1991, 1995, Bengston and Dornhaus 2014; bees: Dreller and Tarpy 1999, Wray, Mattila et al. 2011; wasps: Judd 1998, London and Jeanne 2003). However, behavioural phenotype may be predicted by previous developmental stages. For example, the boldness of a *Polistes metricus* queen during the founding (juvenile) phase of colony development can be used to predict colony aggressive behavioural type (Wright, Skinker et al. 2017).

Vespula wasp colonies are initiated in the spring by a solitary foundress, and can produce thousands of workers before reproducing at the end of a year (Greene 1991; Barlow, Beggs et al. 2002). During the growth phase (mid-summer), the queen remains inside the nest while her offspring (workers) forage and defend the nest; the brood:worker ratio is highest. Finally, during the mature phase (late summer, early autumn), the number of workers reaches a peak, the brood:worker ratio begins to decline, and reproductive brood (new queens and males) are reared. Throughout their growth and mature phase of colony development, *Vespula* colonies exhibit consistent differences in activity and aggression, though neither are correlated with the other (Jandt, Detoni et al. 2020). However, it is unclear whether developmental factors such as the stage of colony development or experience with aggressive stimuli play a role in determining these differences. While individual aggression in *Vespula* has been shown to be influenced by worker age and previous experiences (Santoro, Hartley et al. 2015), individual phenotypes do not necessarily translate directly to the colony level (Pinter-Wollman 2012). Whether colony behaviours (aggression) are subjected to a similar variation to those of individuals is a question of relevance for the understanding of social organization (Jandt, Bengston et al. 2014).

The annual cycle, consistent colony differences in activity and aggression, and the opportunity to manipulate the frequency with which colonies experience a simulated predator attack provide a unique opportunity to explore how development and experience influence variation in aggressive phenotype observed among *Vespula* colonies. Here, we explore how colony level aggression changes throughout colony development, and whether repeated experience with a simulated predator leads to habituation with the stimulus. To do this, we monitored *Vespula* colony response to high or low repeated interactions with a simulated

predator attack throughout the growth and maturation stages of colony development. If colony-level aggression changes over time, colonies should increase or decrease their nest defence behaviour over time. Furthermore, if colonies become habituated by repeated experience to a simulated threat, colonies that experience high repeated interactions should decrease nest defence more rapidly than those colonies with low repeated interactions. On the other hand, if colonies become sensitized to simulated threats, we expected colonies with high repeated interactions to increase nest defence more rapidly than those with low repeated interactions.

3.3 MATERIAL AND METHODS

3.3.1 Study Area and Period

Our study was carried out at the Dunedin Botanic Garden in Dunedin, New Zealand (-45°51'27.59" S, 170°31'15.56" E; 25-85 m elevation). The 30.4 hectare area is urban, open to public visitation and is comprised of varied vegetation that ranges from flower gardens and open grass patches to densely wooded terrain cut by walking tracks (Fig. 3.1). The search for wasp colonies started in February 2018, during the austral summer. Trials took place between March and April 2018. The nests used in the trials were excavated by May 2018 (Appendix: Table 4).

3.3.2 Wasp Colonies

We scanned for *Vespula* colonies both near the tracks and within vegetation. Colonies were located by the wasp activity near the nest opening. Colonies found near areas with high human traffic were not included in this study for safety reasons. We originally identified 13 colonies, but two ceased their activity due to unknown reasons over the course of the experiment (between weeks 1 and 2; see below), and were thus excluded from our data. The final sample consisted of 11 colonies (A-K; Appendix: Table 4), all of which were underground with a single entrance. Every nest found was located on a slope facing either an open area or a walking track.

Vespula colonies in New Zealand are usually initiated during early summer (December/January), with the growth phase of colony cycle happening from January to March/April and the mature phase (end of cycle) happening in April/May (Barlow, Beggs et al. 2002). Temperate colonies of *Vespula* wasps normally show very strict colony cycle seasonality, which leads to a certain degree of synchronism in cycle stages within a wasp population (West-Eberhard 1969; Ishay, Levanon et al. 1983; Godfrey 1995). This means colonies located late February/ early March were in their early growth phase (when wasp activity is reaching its peak in the cycle), and that trials were carried out through the transition from the growth to the reproductive phase (when colonies start to decline).

Figure 3.1. *Vespula* nest locations at the Dunedin Botanic Garden (main aerial map) and location of the Dunedin Botanic Garden within New Zealand (lower left; Botanic Garden location in red). Grey markers indicate colonies in the "hi-freq" treatment group (A-E), while white markers indicate colonies in the "lo-freq" group (F-K). Aerial map sourced from the LINZ Data Service and licensed by Otago Regional Council, for re-use under the Creative Commons Attribution 4.0 International license

3.3.3 Nest Defence Trials

Colonies were randomly assigned into two different treatments: low frequency disturbance (lo-freq) and high frequency disturbance (hi-freq). Lo-freq nests (n = 6) experienced three simulated mammalian disturbance trials (weeks 1, 4 and 7), while hi-freq nests (n = 5) experienced six disturbance trials (weeks 1-6). Although the experiment was carried out in a non-controlled environment, it can be assumed that the only mammalian disturbance these maturing colonies experienced was during the simulated mammalian disturbance we presented during nest defence trials. This is due to the following reasons: (1) New Zealand has no recorded evidence of wasp colony level predation, nor are the primary predators of mature *Vespula* colonies found in New Zealand (Chapter 2); (2) the area goes under regular efforts to eliminate mammalian pests which might interfere with the experiment (e.g. rabbits, stoats, rats); and (3) the nests chosen were not directly accessible by the general public (at least 3 m from walking tracks), and the Botanic Gardens staff was instructed to avoid interacting with colonies. Trials were always carried out between 09:00h and 15:00h (when wasps are most active during the day; Raveret Ritcher 2000) and in days with clear skies (avoiding rain and/or strong winds) to minimize environmental biases. Trials were carried out in a randomized order for every round.

Before a disturbance trial began, a target was set up approximately 1 m away from the nest and 1.5 m from the ground, attached by a thin bamboo stick taped to a plastic tripod. The target was made from two black plastic dishes (18 cm diameter) clasped together, forming a hollow drum (Jandt, Detoni et al. 2020; modified from McCann, Moeri et al. 2015 and Jeanne 1981; Fig. 3.2). The plates housed an omnidirectional condenser microphone (Digitech Audio® AM-4013), attached to a video camera (Panasonic® DMC-FZ300). The video camera was set up to record nest entrance activity and aggressive behaviour directed at the target. Air temperature and air humidity were measured within 1.5 m of the nests using a thermo hygrometer (Temptec® Temptec 4 In 1) for each colony at the start of every trial.

As soon as the target and camera were in place, baseline colony activity (number of forager departures over 4 min) was recorded by video. Following this, we placed the end of a 1.5 m transparent plastic tube (2 cm internal diameter) into the nest entrance and one of the researchers exhaled into the opposite end three times (Jandt, Detoni et al. 2020). The CO_2

Figure 3.2. Experimental set up for *V. vulgaris* nest defence assays in the Dunedin Botanic Gardens, Dunedin, New Zealand. The nest opening is indicated by the pink circle.

and volatile organic compounds (Buszewski, Kesy et al. 2007) provided to the colony by this method simulate the breath of large mammal predators, which are the most common threat to mature temperate vespine colonies (Chapter 2). This ensured that colonies were given a consistent and reliable stimulus to react aggressively (McCann, Moeri et al. 2015; Jandt,

Detoni et al. 2020). We continued recording for two minutes, after which we removed all the materials and moved to the next nest. All materials were cleaned in between trials. For consistency purposes, the same researcher did the exhaling in every trial.

Table 3.1. Ethogram of *Vespula* colony-level behaviours before and after exhaling CO_2 into the nest entrance. A. "Quantified Behaviours" were recorded for hypothesis testing, and analysed in this study. B. "Other Observed Behaviours" include colony behaviours observed after the simulated disturbance, but did not occur often enough to be included in the analyses.

A. QUANTIFIED BEHAVIOURS	
Behaviour name	Description
Pre-disturbance Foraging Activity	Wasps exit the nest entrance in a straightforward flight path.
Target Strike	Wasps strike the target set at 1 m from the nest.
Defence Flight	Multiple wasps exit the nest following the disturbance and fly around the nest entrance in one of two patterns: 1. wasps fly swiftly in wide arcs (≤ 2m from entrance); 2. wasps hover around the nest opening (≤ 30cm from entrance).
B. OTHER OBSERVED BEHAVIOURS	
Retreat	Wasps previously engaged in "Defence Flight" return to the nest in large groups, usually marking the end of Defence Flight.
Non-Target Strike	Wasps strike objects other than the target, such as tree roots, rocks, plants, the recording equipment, or the researchers.
Guarding	Wasps station themselves outside the nest opening and face the surrounding environment with raised antennae. This behavior can last for > 120s after simulated disturbance.
Wasp Aggression	Often "Guarding" wasps, these individuals tackle other wasps approaching the nest entrance mid-flight. Interactions last for < 1s, after which the attacker resumes its previous activity.
Disturbed Activity	After "Defence Flight" ends, wasps fly in and out of the nest opening (similar to Baseline Activity), but briefly interrupt their flight (≤ 1s) to hover near the nest entrance, often antennating other nearby wasps mid-flight. This behavior can last for > 120s after simulated disturbance.

Using J-watcher (version 1.0, jwatcher.ucla.edu), we quantified baseline activity and the amount of time colonies engaged in defence flight (post-disturbance); target strikes (pre-

and post-disturbance) were quantified in J-watcher using sound files from the target's microphone (see Table 3.1 for definitions of behaviours). Other behaviours observed at the nest (Table 3.1) did not occur at a high enough frequency to be quantified for analysis. Observers were blind to the treatment when collecting behavioural data from videos.

3.3.4 Nest Measurements

V. vulgaris colonies in this experiment were all underground, making non-invasive nest measurements over the course of the study impossible. Of the 11 colonies, seven could be excavated by the end of the experiment (4 hi-freq, 3 lo-freq). Colonies were anesthetized with diethyl ether, and nests were excavated (Appendix: Table 4). Wasps were collected from each nest to confirm species identification. Combs were separated and photographed with an object for scale. Images were analysed in ImageJ, version 1.0.8_112 (Schneider, Rasband et al. 2012). Because colonies had reached (or passed) the mature phase by the time they were excavated, combs had very few brood left inside. Therefore, we quantified colony investment into the nest as the total comb surface area.

3.3.5 Statistical Analysis

All statistical analyses were conducted in R, version 4.0.3 (R Core Team 2020). To verify the effect of the different variables on colony behaviours, we used mixed-effect models with 'colony ID' as a random factor (α=0.05), using the package 'lme4' (Bates, Maechler et al. 2005). When interaction terms between covariates were included in models but found not to be significant, those interactions were removed and new models were generated. Type III ANOVAs were used to assess the significance of the relationships in the models. To balance the data, all comparisons were made using hi-freq colony data from weeks 1, 4 and 6; and lo-freq colony data from weeks 1, 4 and 7 (all weeks), and treating week as a categorical factor ("first", "middle", or "last"). Data for weeks in the hi-freq treatment that were not included in the analysis are presented in the Appendix (Supplementary Fig. 3).

Before nest defence behaviours could be tested, we looked for statistical relationships between environmental variables and time in order to avoid including covarying factors in

the same model. To do this, we built linear mixed-effect models (LMMs) with: (1) temperature as the response variable and week as a factor, and (2) humidity as the response variable and week as a factor. Both response variables (temperature and humidity) were normally-distributed, continuous data (see below). Because there was a significant relationship between temperature and week (see 'Results'), week was considered to represent the variation in temperature data in further models.

To measure the effects of covariates on pre-disturbed foraging activity (the number of forager departures recorded in 4 minutes), we used generalized linear mixed-effects models (GLMMs) with a Poisson distribution and log-link function. These GLMMs were built with pre-disturbance foraging activity as the response variable and (1) treatment, week, and their interaction as factors; and (2) treatment as a factor and temperature as a covariate. Since activity was the response variable on both models, we used a Bonferroni correction (m=2, confidence interval=0.975). Post hoc estimated marginal means (EMM; 'emmeans' package, Searle et al. 1980) were used on the models to look for significant pairwise differences within categorical data (week). Because activity covaried significantly with both environmental factors (temperature and humidity) and week (see 'Results'), in further models activity was included separately from those variables, and considered to represent their variation.

To verify the effect of variables on nest defence behaviour, we first used LMMs with defence flight duration (normally-distributed, continuous data; see below) as the response variable. The models were built with: (1) treatment, week, and their interaction as factors, and humidity as a covariate; and (2) pre-disturbance foraging activity as a factor. Since defence flight was a response variable on both models, we used a Bonferroni correction (m=2, confidence interval=0.975). Post hoc estimated marginal means (EMM) were used on the models to look for significant pairwise differences among categorical variables (weeks). In addition to defence flight, we used GLMMs with a Poisson distribution and log-link function to examine nest defence behaviour by using target strikes as the response variable. These models included: (1) treatment, week, and their interaction as factors, and humidity as a covariate; (2) pre-disturbance foraging activity as a factor; (3) defence flight duration as a factor (to determine whether the two proxies were correlated). Since target strikes was a

response variable on all three models, we used a Bonferroni correction (m=3, confidence interval=0.983).

Finally, to determine whether nest size could be used to predict defence flight duration, we ran a Linear Model (LM) built with the defence flight duration data from the last trial for each colony that could be excavated (week 6/trial 6 for hi-freq, week 7/trial 3 for lo-freq). Defence flight was included as the response variable, and nest size (m^2), treatment, and their interaction as factors. Colonies whose nests could not be excavated (n = 4, Appendix: Supplementary Table 4) were excluded from this analysis.

For all models, p-values were obtained using the package 'lmerTest' (Kuznetsova, Brockhoff et al. 2017). Degrees of freedom estimates for individual effects in GLMMs were calculated using the package 'MASS' (Venables and Ripley 2002). Normality of response variables were graphically verified with Q-Q plots, using the package 'ggpubr' (Kassambra 2020). The distribution of the model's residuals was used to further confirm the assumption of normality for the response variables. Descriptive statistics shown in the results are depicted as 'median ± confidence intervals'.

3.4 RESULTS

Behaviours pre- and post-disturbance during trials are defined in Table 3.1. A summary of model outputs is provided in the Appendix (Supplementary Table 5).

3.4.1 Abiotic Factors

Temperature measured around the nest during trials decreased significantly over time (week: $F_{2,30}$=5.536, p=0.009). Therefore, the inclusion of 'week' in later models was considered to account for the effects of temperature variation on the data. Humidity varied independently of time (week: $F_{2,30}$= 0.958, p=0.395).

3.4.2 Pre-disturbance behaviour

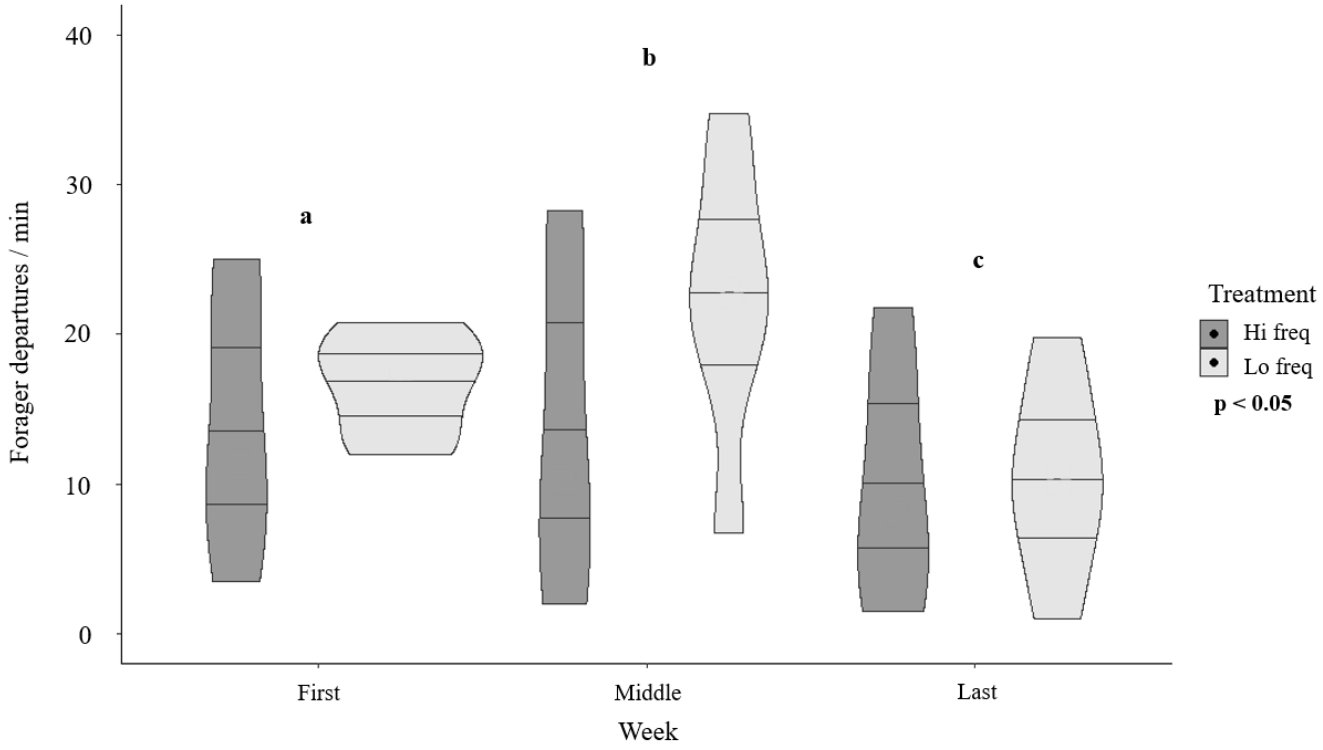
During trials, all wasp colonies were engaged in pre-disturbance foraging activity (15±2.99 forager departures/min; Fig. 3.3). When humidity was included in the model, we found evidence that foraging activity decreased over time and as humidity increased, but was not affected by treatment (week: $F_{1,19}$=51.94; p<0.001; treatment: $F_{1,9}$=1.357; p=0.274; humidity: $F_{1,19}$=7.656; p=0.010; Fig. Fig. 3.3). When temperature was included in the model, foraging activity was again not affected by treatment but did increase with temperature (treatment: $F_{1,9}$=1.355; p=0.274; $F_{1,21}$=72.711; p<0.0001; Fig. 3.3). Foraging activity levels can be used as a proxy for colony size (Malham et al. 1990), so we include pre-disturbance activity in our statistical models as colony size likely changed over time. However, due to its interaction with other measured variables, later models including pre-disturbance activity do not include temperature or humidity. None of the colonies engaged in pre-disturbance defence flight, similar to the results in Jandt, Detoni et al. (2020).

3.4.3 Behavioural Response to Disturbance: Defence Flight

We observed post-disturbance defence flight in most colonies and trials (81.8% of all colonies x trials; Fig. 3.4). When defence flight concluded within the two-minute trial, the colony shifted to disturbed activity or baseline activity (Table 3.1). When colonies did engage in post-disturbance defence flight, it occurred within the 30s immediately following disturbance (response delay=3±1.879s).

Overall, defence flight duration decreased over time, but was not affected by disturbance frequency or relative humidity (week: $F_{2,19}$ =4.697; p=0.022; treatment: $F_{1,9}$=0.109; p=0.749; humidity: $F_{1,23}$=1.504; p =0.232; Fig. 3.4i). The duration of defence flight was significantly lower in the last week compared to the first week (EMM: $p_{first:last}$=0.022), the middle week did not differ (EMM: $p_{first:mid}$=0.175; $p_{mid:last}$=0.925). No significant effect on defence flight duration was found when pre-disturbance foraging activity was included in the model (activity: $F_{1,27}$=0.262; p=0.613; Fig. 3.4ii).

i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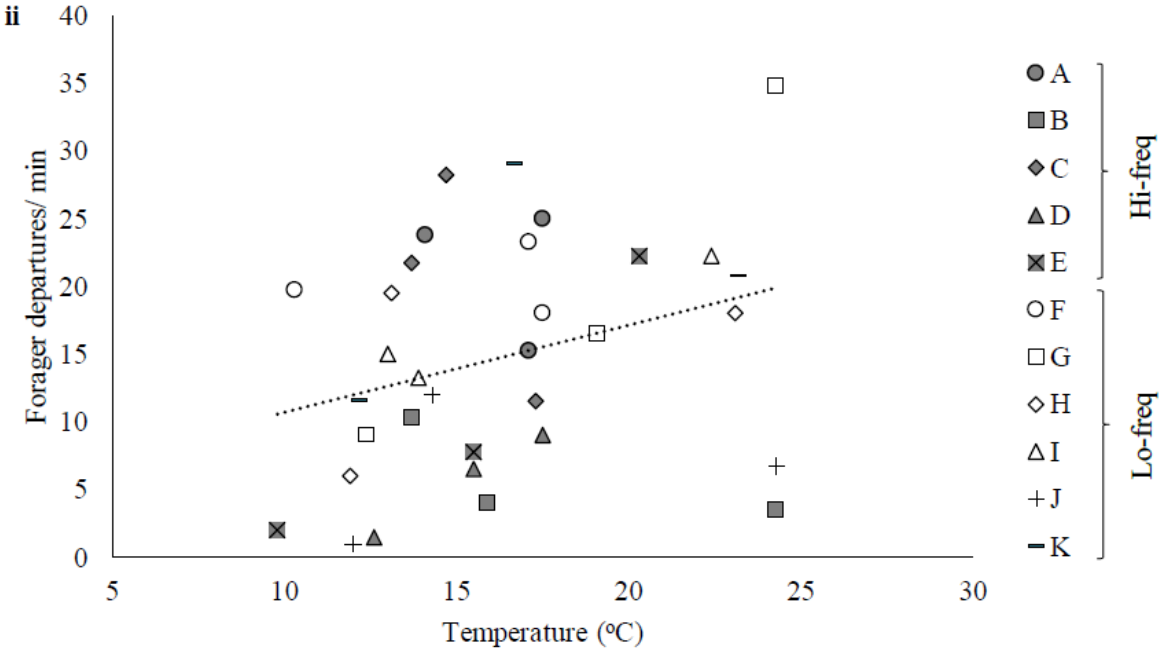
ii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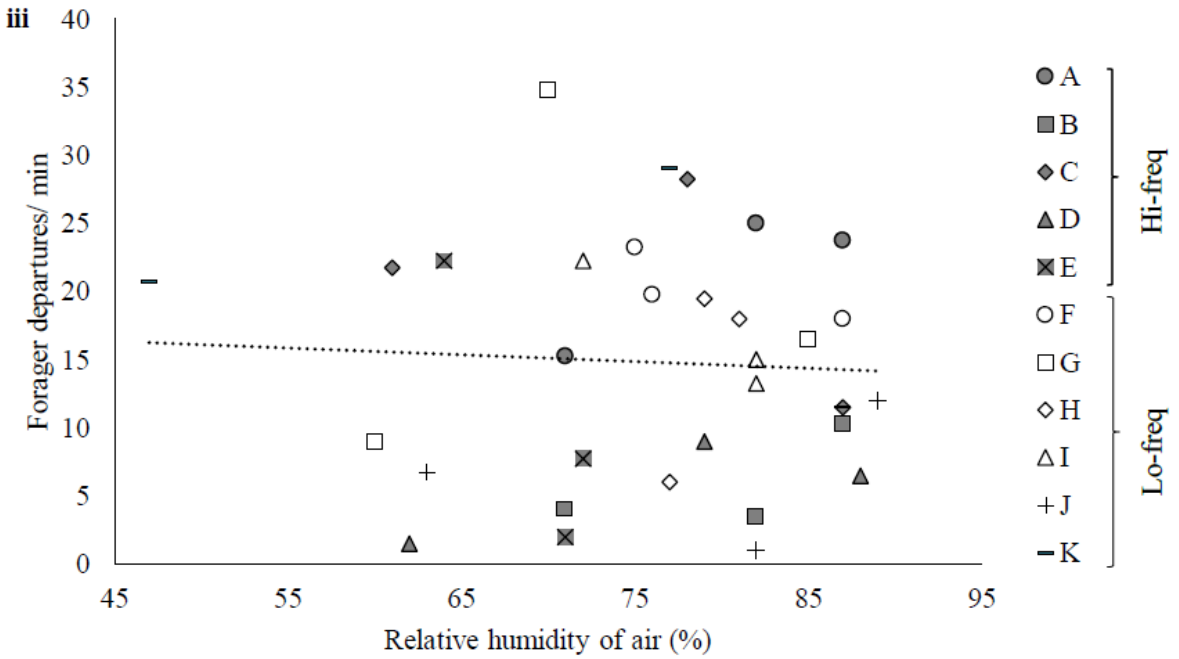

Figure 3.3. Effects of time and abiotic factors on colony pre-disturbance foraging activity across treatments and colonies. (i) Distribution of forager departures per minute across first, middle, and last weeks. Horizontal lines within violin plots represent quartile and median distribution of defence flight duration, while plot width represents frequency density. Weeks with different letters were statistically different (p=0.01). (ii) Air temperature and (iii) air humidity measured over six/seven weeks for each nest were significant predictors of forager departures ($p \leq 0.01$). Each colony is represented by a different symbol/colour combination. For all figures, hi-freq colonies are represented as filled boxes/symbols; lo-freq as white boxes/symbols.

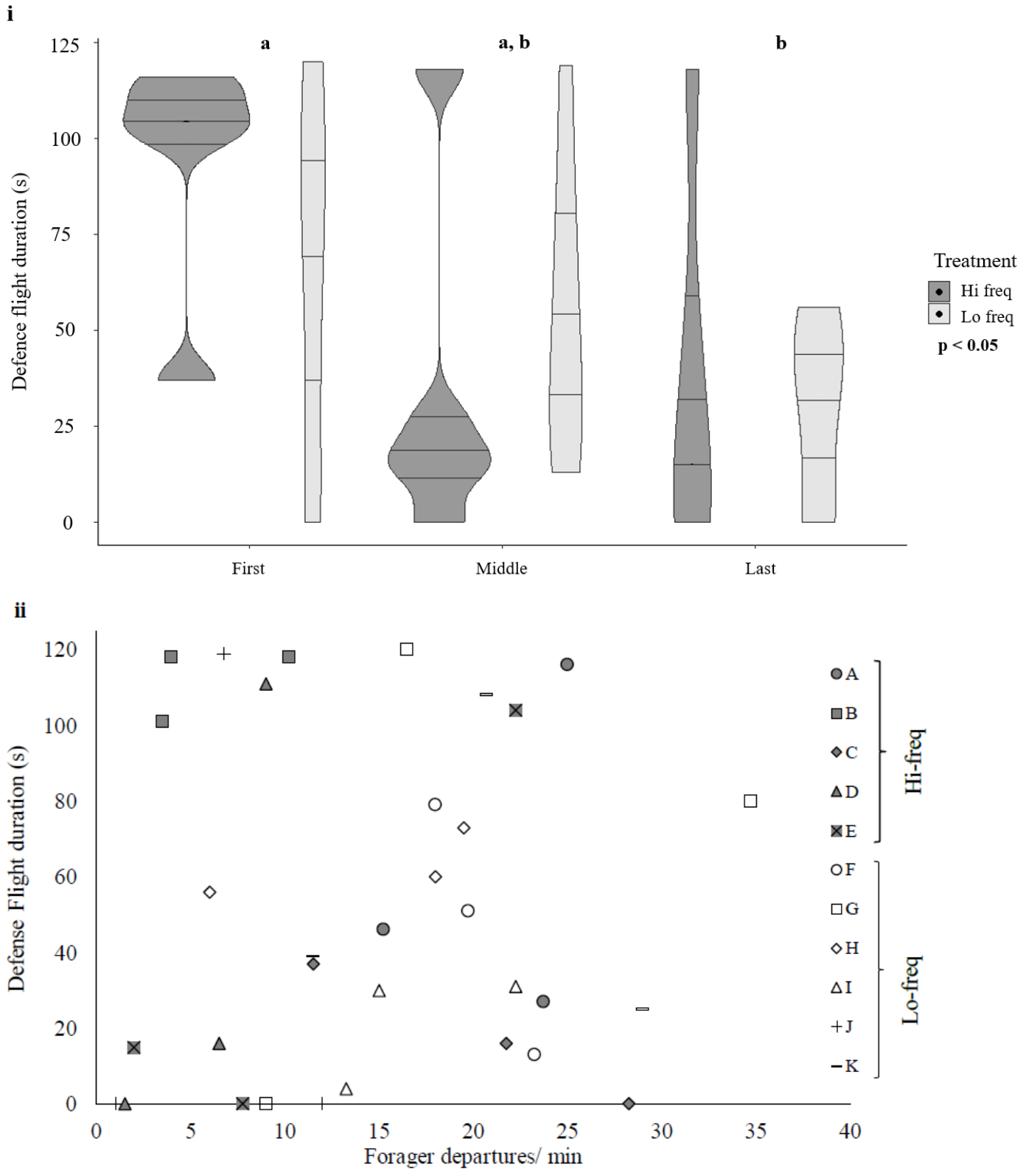
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Figure 3.4. Effects of time and pre-disturbance foraging activity on colony defence flight duration (s) across treatments. (i) Distribution of defence flight duration across weeks (hi-freq: weeks 1, 4 and 6; lo-freq: weeks 1, 4 and 7). Horizontal lines within violin plots represent quartile and median distribution of defence flight duration, while plot width represents frequency density. Week is a significant predictor of defence flight duration ($p=0.021$; weeks with different letters are statistically different at $p<0.05$). (ii) Post-disturbance defence flight duration could not be predicted based on pre-disturbance forager departures ($p > 0.05$). Filled markers represent colonies in the hi-freq treatment (A-E), while clear markers represent colonies in the lo-freq treatment (F-K).

3.4.4 Behavioural Response to Disturbance: Target Strikes

After disturbance occurred, target strike behaviour was observed only in 30.3% of colonies x trials (Fig. 3.5). As a result, target strike data in this study was mostly comprised of null values (but see Jandt et al. 2020). Still, we found a positive correlation in that colonies that spent more time displaying defence flight engaged in significantly more target strikes (defence flight: $F_{1,21}=50.759$; $p<0.001$). However, we found no evidence that time (week: $F_{2,19}=3.022$; $p=0.072$; Fig. 3.5i), frequency of disturbance (treatment: $F_{1.9}=2.066$; $p=0.184$), or relative humidity (humidity: $F_{1,19}=0.829$; $p=0.374$) were significant predictors of target strikes after a disturbance. Interestingly, colonies with higher pre-disturbance activity made more target strikes ($F_{1,21}=50.298$; $p<0.001$), though the slope of this relationship is quite small (Fig. 3.5ii).

3.4.5 Nest Size and Behavioural Responses to Disturbance on the Last Trial

When only data from the last week of trials are compared, we found no evidence that nest size could be used to predict defence flight duration (nest size: $F_{1,4}=1.06$; $p=0.361$; treatment: $F_{1,4}=0.163$; $p=0.707$; Fig. 3.6).

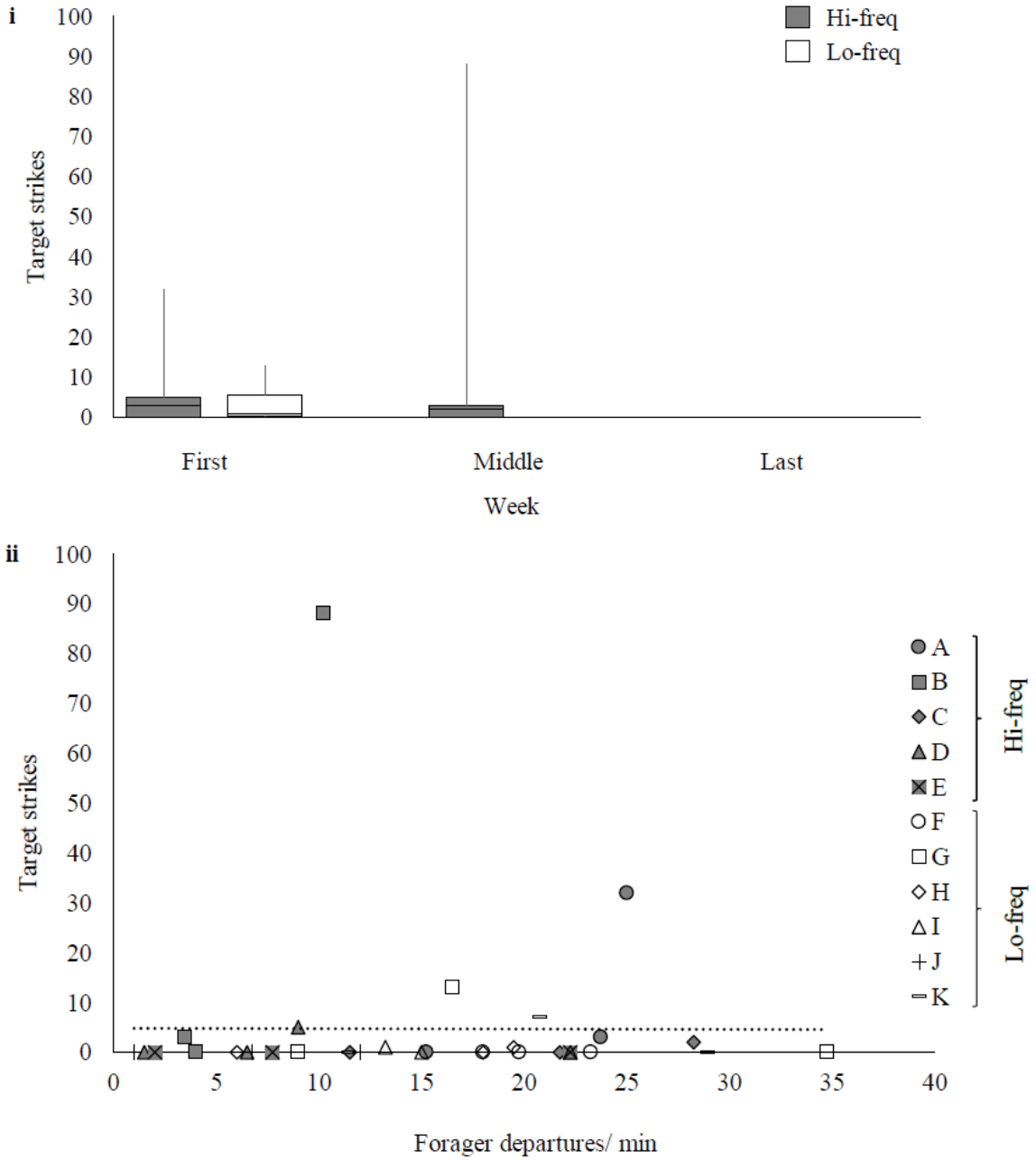

Figure 3.5. Effects of pre-disturbance foraging activity on colony target strikes across treatments. (i) Boxplots showing the distribution of defence flight duration across weeks (hi-freq: weeks 1, 4 and 6; lo-freq: weeks 1, 4 and 7). Boxes represent quartile and median distribution of defence flight duration, lines, minimum and maximum values. Filled bars represent colonies in the hi-freq treatment, while clear bars represent colonies in the lo-freq treatment. No significant relationship was found between variables ($p>0.05$). (ii) Scatterplot showing distribution of defence flight duration vs. forager departures before disturbance. Filled markers represent colonies in the hi-freq treatment (A-E), while clear markers represent colonies in the lo-freq treatment (F-K). Forager departures are a significant predictor of target strikes (GLMM's type III ANOVA: $F_{1,30}=50.298$; $p<0.01$).

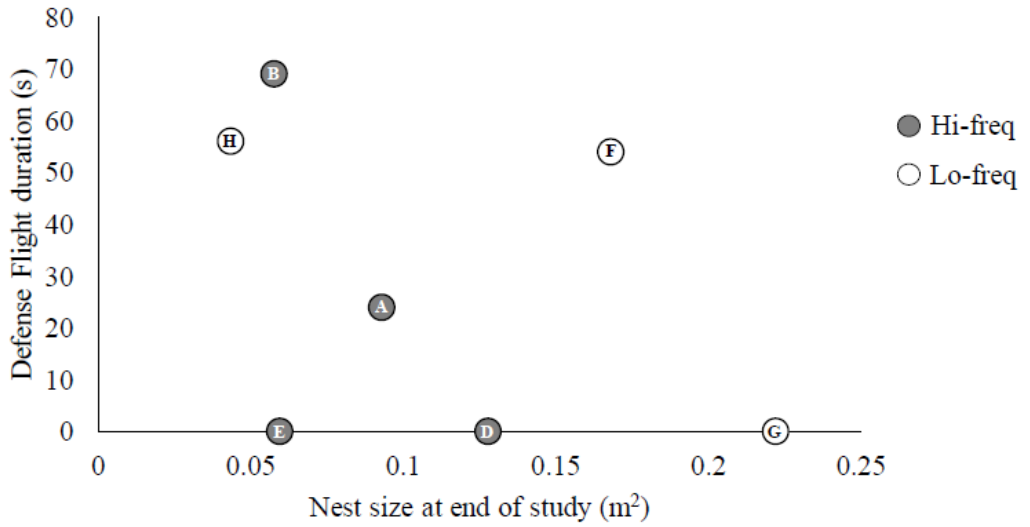

Figure 3.6. Nest size (m^2) at end of study and last recorded defence flight duration (s) across excavated colonies. Hi-freq colonies (n=4) are represented by grey markers, while lo-freq (n=3) are represented by white markers. Colony ID is included within markers. No significant relationship was found between variables ($p>0.05$)

3.5 DISCUSSION

Here we show that the aggressive response of *V. vulgaris* colonies to simulated mammalian disturbance decreases as colonies mature from their growth into their reproductive phase of colony development. By subjecting half our colonies to high and low frequency experiences, if this decrease were due to habituation with a non-threatening simulated disturbance, the colonies in our high-frequency disturbance treatment group should have decreased their response more quickly than those in the low-frequency group (i.e., during the 'middle' week). Instead, colonies in both treatment groups reduced their response to the disturbance at relatively the same rate over time. Additionally, we show that a colony's pre-disturbance foraging activity also decreased over time, suggesting that colony size (number of adults) was also decreasing (Malham, Rees et al. 1990) and was affected by abiotic factors (temperature and humidity). However, similar to previous work on *Vespula* colony defensive behaviour (Jandt, Detoni et al. 2020), we found no evidence that foraging activity could be used to predict aggressive nest defence of wasp colonies.

A large wasp colony, i.e., one with more workers, could react more intensely to a threat or disturbance, simply because it can deploy more defenders. For example, honey bee

colonies tend to behave more aggressively as they grow (Schneider and McNally 1992), and the outcome of intraspecific territoriality conflicts in Azteca ants can be reliably predicted by the size of the colonies involved (Adams 1990). However, we found no evidence to suggest that colony size could predict colony defensive response. Wasp colonies in temperate environments can be highly seasonal and have synchronous developmental cycles (West-Eberhard 1969, Ishay, Levanon et al. 1983). Moreover, *Vespula* colony size can be predicted based on traffic rate (activity moving in and out of the nest; Malham, Rees et al. 1990). That decrease in traffic rate throughout the experiment was expected: data collection ranged from the middle to the end of the typical wasp season in New Zealand (March-May), and a decrease in foraging activity towards winter is the norm in temperate wasp species (Barlow, Beggs et al. 2002). In both our measures of colony size (pre-disturbance activity and nest size), our results support those found in previous studies on *Vespula* in that larger colonies are not more or less aggressive than smaller colonies.

Defensive behaviours can change over time at the individual level, whether due to habituation to a stimulus or developmental changes. For instance, older mice tend to be less aggressive towards conspecific intruders (Engellenner, Burright et al. 1986); meanwhile, in burrower bugs, experienced mothers spend less time engaged in active brood protection (Kight 1997). In social insects, individuals tend to get more aggressive over time, a phenotype linked with physiological and genomic changes (ants: Wilson 1971; Kühbandner, Modlmeier et al. 2014; social bees: Breed, Robinson et al. 1990; Paxton, Sakamoto et al. 1992); and wasps: Jeanne, Williams et al. 1992; Togni and Giannotti 2010; Monceau, Bonnard et al. 2013, including *Vespula* wasps: Santoro, Hartley et al. 2015). Evolutionarily, older individuals may be more expendable, and more useful to the colony as guards or defenders (Jeanne, Williams et al. 1992).

Change in individual-level aggression may not, however, translate directly to change in colony-level aggression (Pinter Wollman 2012). Worker lifespans are considerably shorter than their colony's (e.g. *Vespula* workers live for 2-3 weeks, while the colony often lasts for more than 6 months; Greene 1984). Therefore, older colonies are not necessarily comprised of older workers. In fact, it has been shown that in the Neotropical social wasp *Polybia occidentalis*, colonies with older workers are not necessarily more aggressive (London and Jeanne 2003).

The brood:worker ratio, which peaks in the middle of the colony cycle, has been proposed as a causal mechanism to explain variation in colony-level aggression over time (London and Jeanne 2003; Judd 1998). In Neotropical social wasp colonies, nest defence efforts grow steadily from the colony's foundation to the point at which brood:worker ratio is maximized. From an evolutionary perspective, the risk of fighting a predator is offset by the colony's increase in reproductive investment. Towards the end of the colony cycle, following the production of gynes (virgin queens; Greene 1991), defensive behaviour also begins to decrease (Judd 1998; Monceau, Bonnard et al. 2013). This coincides with the low levels of aggression we show for the last weeks of trials, and with minimal brood in nests we found after excavation - some colonies were completely devoid of them. Conversely, other investigations in the Neotropics have not been able to verify the effect of brood:worker ratio on the colony's nest defence behaviour (Seal 2002). Furthermore, when tested mid growth phase, previous work on *Vespula* colony aggression found no evidence that proportion of brood in a colony could be used to predict defensive behaviour (Jandt, Detoni et al. 2020).

As wasp colonies shift from their growth to reproductive phase, a variety of changes are happening inside the colony, including change of brood:worker ratio. The *Vespula* queen typically lives for one year, and the loss of the queen can trigger colony decline (Greene 1991), which is likely what happened to two of the colonies in this study that did not last the full 7 weeks. A colony without a queen may still retain some reproductive potential (Landolt, Akre et al. 1977; Greene, Akre et al. 1978), but the colony's social cohesion tends to decline steadily after the queen is gone (Landolt, Akre et al. 1977). Monceau, Bonnard et al. (2013) suggest that nest defence is mediated by the presence of the queen, and its loss could result in decreased aggressiveness towards external threats. Towards the end of the reproductive phase, vespine colonies begin to produce male wasps and gynes, which can make up a significant part of the adult population (Ishay, Levanon et al. 1983). These reproductive individuals typically do not perform tasks around the nest, are unable to sting, and would have very little incentive to engage with a predator (Hamilton 1964; O'Donnell 1995).

We also found evidence that abiotic factors are linked to colony behaviour. Temperature and humidity could reliably predict pre-disturbance (or undisturbed) foraging activity, which is well documented for various social wasp taxa (Raveret Richter 2000; Detoni and Prezoto 2021), as well as other social insects (Burrill and Dietz 1981). In some

social insects, abiotic factors can influence aggression behaviour; for instance, nest defence in honey bees may be influenced by temperature and air humidity (Southwick and Mortitz 1987). Although no direct relationship was found between temperature and air humidity on nest defence, in seasonal nesting species such as temperate *Vespula*, shifts in environmental variables by the end of the cycle in late autumn coincide with a decrease in the availability of food resources (Potter 1964). Thus, it is possible that starvation at colony-level can directly inhibit risk prone, energetically-expensive behaviours such as defence flight. Finally, in an indirect way, older and poorly-fed social insect colonies are more prone to parasitic infestations (Evans and Schwarz 2011); the physiological stress caused by high viral loads at the colony level could act as inhibitors of costly aggression-related behaviours.

Because of the strong effect of time, we found no evidence that *Vespula* colonies habituated to the simulation of a mammalian intruder. Still, although no significant statistical difference between treatments was found, defence flight duration differed visibly between lo- and hi-freq colonies on the first week. This was not expected, and possibly a random effect of the high variability of the behaviour across colonies. However, both treatments behaved similarly over time, with converging phenotypes on the last week. This supports the significant effect of time shown in the model, but not any of the predicted effects of experience. Colony-level habituation to non-threatening stimuli has been shown for honey bees – that is, the more often the bees are disturbed, the less likely they are to attack a non-harmful target (Rittschoff and Robinson 2013). It is possible that our experimental design did not provide enough of a difference in terms of how frequent simulated threats were provided to colonies between treatments. In this case, if habituation was present, both treatment groups could have been affected similarly, with no observable difference between their colonies. An experimental protocol including an even higher frequency of disturbances for the hi-freq group (e.g., twice a week) could help address this issue. However, the logistic constraints of doubling the sampling effort over time, in addition to the limited seasonal availability of nests and the need for ideal weather conditions for the experiment made this change unfeasible in our study. Furthermore, it is worth noting other studies were similarly unable to verify the effect of experience on nest defence over time on social wasp colonies (*Polybia occidentalis*; London and Jeanne 2003), which strengthens the biological validity of our results.

Based on the significant effect of time variation on defence flight, in contrast with every other variable analysed, it is likely that nest defence in social wasps has very little behavioural plasticity. A colony's willingness to aggressively react towards a perceived threat is likely to be strongly dictated by a few key intrinsic influences. For instance, on top of the colony social-demographic mechanisms discussed above, we can add the possibility of aggression-related hereditary mechanisms operating at colony level (Wright, Skinker et al. 2017; Manfredini, Brown et al. 2018). Whether *Vespula* colony aggressiveness can be determined by its gene pool and how would that translate to over-time behavioural variation is a question that could be addressed in future investigations.

Target strikes have been used to gauge colony aggression in previous studies (McCann, Moeri et al. 2015: *V. pensylvanica*, *V. alascensis*, and *V. germanica*; Jandt, Detoni et al. 2020: *V. germanica* and *V. vulgaris*). However, in our study, target strikes were rarely observed. Jandt, Detoni et al. (2020) highlighted the contrast between colony level aggression in *V. germanica* and *V. vulgaris*, particularly that the latter species exhibits substantially lower levels of aggression. As a result, we described and investigated time spent engaged in defence flight – as that provided data with variation more appropriate for statistical testing. Moreover, we confirm that colonies that engaged in more defence flight were also more likely to strike the target, suggesting that both aggression proxies can be used to quantitatively describe the aggressive phenotype of the colony.

3.6 CONCLUSION

Behavioural phenotypes in animals can change both as individuals and/or colonies mature (Bengston and Jandt 2014). Internally, individuals change as they age, but so do colonies. Over time, colonies shift through demographic ratios, reproductive viability of the queen, increase of parasites/disease, and individuals change in terms of reproductive potential (Jandt and Dornhaus 2011). Although colonies are comprised of aggressive and less-aggressive individuals (Kühbandner, Modlmeier et al. 2014; Santoro, Hartley et al. 2015) because of the interactions with the social and external environment, colony aggression phenotypes are likely determined by more than just the sum of aggressive and non-aggressive individuals (Pinter-Wollman, Hubler et al. 2012b).

Here, we show that nest defence behaviours decrease over time, occurring concomitantly with the maturation of colonies towards the end of their life cycles. Although we could not observe a direct effect of experience on nest defence, colonies may still habituate to some degree to non-threatening disturbance, especially over shorter periods of time (see supplement in Jandt, Detoni et al. 2020).

There are likely intrinsic effects controlling the variability of aggressiveness across *V. vulgaris* colonies. Still, it is unclear what or how ecological factors may be affecting nest defence behaviours. Does colony aggression differ across geographic and/or native and introduced ranges? Moreover, what are the genomic mechanisms affecting aggressive behaviour in *Vespula*, and how do they shift as the colony develops? There is a large avenue open for future investigations, which in turn can improve our understanding of the ecology and evolution of eusocial organisms.

Chapter Four

Brain Gene Expression Reveals Limited Caste Differentiation between Foragers and Defenders in a Social Wasp[4]

4.1 ABSTRACT

The organisation of workers in behavioural castes occurs in most social insect societies. These behaviourally specialized individuals perform a set of tasks in the colony; younger, less aggressive workers tend to perform in-nest tasks such as nursing, whereas older, more aggressive workers are usually in charge of out-of-nest tasks such as predator defence. Different genetic mechanisms have been suggested to explain variation in worker aggression across ecological contexts, yet a small number of studies have focused on explaining the role of gene expression in behavioural specialization during nest defence. Here, we compared the gene expression patterns in brains of nest defenders versus foragers of the social wasp *Vespula vulgaris*, aiming to identify a transcriptomic basis for aggression in the context of nest defence. Three *V. vulgaris* colonies were studied in Dunedin, New Zealand. Colony phenotypes were obtained through field-based behavioural assays. Foragers and defenders were collected from each colony, and brain gene expression was analysed through next gen RNA-seq. Our results show weak differential gene expression between the two behavioural castes, despite the phenotypical and transcriptomic differences observed among colonies. We provide evidence for limited caste differentiation between out-of-nest workers in *V. vulgaris*, which aligns with the low genetic diversity within colonies and overall weak division of labour described for the species. The brain transcriptome of *V. vulgaris* is described for the first time. We provide further discussion on the biological significance of the genes found to be differentially expressed between foraging and defensive behavioural castes, and compare these results with previous investigations on the transcriptomic mechanisms underlying task partitioning in social insects.

Key words: Aggression; Hymenoptera; nest defence; transcriptomics.

[4] This study was carried out in collaboration with co-authors Peter Dearden (Department of Biochemistry, University of Otago, New Zealand), Ludovic Dutoit (Department of Zoology, University of Otago, New Zealand), Tom Harrop (Department of Biochemistry, University of Otago, New Zealand), Joanna Ward (Department of Zoology, University of Otago, New Zealand), and Jennifer Jandt (Department of Zoology, University of Otago, New Zealand).

4.2 INTRODUCTION

Behavioural castes occur nearly ubiquitously in insect societies (e.g., ants, bees, wasps, and termite colonies) (Gordon 2016). Groups of individuals within each caste consistently perform a set of tasks such as foraging, nursing, or defending the nest (Oster and Wilson 1978, Robinson and Jandt 2020). Behavioural specializations can be highly adaptive for the colony because they allow for higher efficiency in task performance (Jandt, Bengston et al. 2014), and division of labour is a main cause of social insect ecological success (Robinson 1992).

Many social insects are known for their effective nest defence strategies. "Nest defence" can be defined as a functional grouping of defensive aggression-related behaviours dedicated to self-preservation and brood protection in social insect colonies (Rittschof and Grozinger 2021). For example, honey bee and wasp colonies exhibit collective-stinging retaliation against predator attacks (Breed, Guzmán-Novoa et al. 2004, McCann, Moeri et al. 2015). Some colonies have groups of workers dedicated to nest defence; these behavioural castes are usually the first to react to a disturbance at the nest site. Nestmates are usually alerted to the potential predator's presence by "guards" (Breed and Rogers 1991, Paxton, Sakamoto et al. 1994, O'Donnell, Hunt et al. 1997, O'Donnell and Jeanne 2002), while "soldiers" are primarily responsible for aggressively engaging with the threat (Breed, Robinson et al. 1990, Breed, Smith et al. 1992).

The execution of consistent behaviours can be determined by genetics, physiology, and neural-hormonal pathways and the environment (Mackay 2014). For instance, honey bee guards can be distinguished from nestmates belonging to other behavioural castes in their genetic makeup and/or gene expression patterns. Defenders may belong to different subfamily groups when compared to other workers, differing in allozyme frequencies (Breed, Robinson et al. 1990), while bees in aggressive-prone cohorts express several genes differentially when compared to other age groups (Alaux, Sinha et al. 2009). Investigating the genomics of social insect castes can provide valuable insights into their division of labour and consistent behavioural differences. Even among highly related workers, different behavioural castes show different patterns of gene expression in the brain (Evans and Wheeler 2000). In monomorphic species, where individuals within behavioural castes are

morphologically similar, omic differences can explain the rise and maintenance of variation in consistent behavioural differences between castes (Smith, Toth et al. 2008, Jandt and Gordon 2016).

Workers of social hymenopteran colonies typically show lower levels of genetic diversity when compared to other animal societies (Hölldobler and Wilson 2008). When reproductive females mate with only a few males, the brood are highly related to each other (Laidlaw and Page Jr. 1984). This makes the eusocial Hymenoptera an ideal model for the study of behavioural transcriptomics since different patterns in gene expression among workers are more likely to be due to non-hereditary mechanisms (Zayed and Robinson 2012). Previous investigations on the transcriptomics of social wasp caste differentiation have been mostly focused on comparing differentially expressed transcripts (DETs) between workers and reproductive castes, rather than within worker behavioural castes (Berens, Hunt et al. 2015, Geffre, Liu et al. 2017). Conversely, few investigations have focused on understanding the omics underlying aggression behaviours in the context of nest defence. A couple of published studies (Alaux, Sinha et al. 2009, Rittschof and Robinson 2013) have provided a good starting point by documenting that gene expression is linked to nest defence behaviour in honey bees, but it is unknown whether this behaviour has similar genetic underpinnings in other social insects. Investigating the genomic bases for behavioural variability in wasps provides further valuable insights into the evolution of social behaviour in Hymenoptera.

Vespula is a genus of social wasps (Vespidae: Vespinae) that shows moderate division of labour amongst workers, which present a certain level of behavioural specialization and consistency (Hurd, Jeanne et al. 2007, Santoro, Hartley et al. 2019). *Vespula* workers tend to shift between tasks as they age; younger workers tend to focus on in-nest tasks, while older ones do external tasks such as foraging and defending the nest (Hurd, Jeanne et al. 2007). Despite this cohort similarity, individual aggression varies (Santoro, Hartley et al. 2015), which may indicate the existence of a behavioural caste of nest defenders.

Here, we compare the transcriptomes of foragers and defenders in *Vespula vulgaris*. We identify genes differentially expressed between the two castes, and compare them with other aggression-related genes found in other animals. We discuss aggression genes linked

to nest defence, as well as their distinct functionality when compared to other ecological aggression contexts.

4.3 MATERIAL AND METHODS

4.3.1 Wasp Colonies

Vespula vulgaris colonies were located in natura at the Dunedin Botanic Garden in Dunedin, New Zealand (-45°51’27.59” S, 170°31’15.56” E; 25-85 m elevation), in 2019. *Vespula* colonies in New Zealand are usually initiated during early summer (December/January), with the growth phase of colony cycle happening from January to March/April and the reproductive phase (end of cycle) happening in April/May (Barlow, Beggs et al. 2002). Based on the typical seasonality of temperate *Vespula* populations, (West-Eberhard 1969, Godfrey 1995), and because the search for wasp colonies started in February 2019 (during the austral summer), we assumed that colonies studied here were found in the growth phase (when wasp activity is reaching its peak in the cycle), and that trials were carried out through the transition from the growth to the reproductive phase (when colonies start to decline).

The study area was scanned for *V. vulgaris* colonies both near the tracks and within vegetation. Colonies were located by wasp activity near the nest opening. Colonies found near areas with high human traffic were not included in this study for safety reasons. Thirteen colonies were originally identified, but due to the nature of the experimental design only those with visible and accessible nest openings were included in our data collection; additionally, one colony ceased activity before the end of data collection period and was also excluded from analyses. The final sample consisted of nine colonies, all of which were underground with a single entrance. Before data collection took place, wasps were collected from each nest to confirm species identification.

From the nine colonies found, three (named L, M, and N) were chosen to be included in this study after the first trial. These colonies were chosen due to exhibiting measurable responses to disturbance (see ‘Colony Phenotypes’ below; also see Supplementary Table 6).

4.3.2 Colony Phenotypes

The aggressive phenotype of *V. vulgaris* colonies was determined experimentally based on their behavioural response to a simulated mammalian threat. Colonies were trialled over three rounds, starting on the week following the collection of biological samples (23/03 - 14/04/2019; see 'Genetic Sample Collection and Preparation' below). Each round took place in a different week, meaning that phenotypes were gauged three times for each colony over the course of three weeks. Because collective nest defence effort in *Vespula* is consistent over short terms (Jandt, Detoni et al. 2020), we expect that the behavioural data collected across three weeks serves as an accurate proxy for the colony's phenotype during the collection of biological samples.

The methods for experimental disturbances were the same as those used in Chapter 3, based on McCann, Moeri et al. (2015) and Jandt, Detoni et al. (2020). Before a disturbance trial began, a target was set up approximately 1 m away from the nest and 1.5 m from the ground, attached by a thin bamboo stick taped to a plastic tripod. The target was made from two black plastic dishes (18 cm diameter) clasped together, forming a hollow drum. The plates housed an omnidirectional condenser microphone (Digitech Audio® AM-4013), attached to a video camera (Panasonic® DMC-FZ300). The video camera was set up to record nest entrance activity and aggressive behaviour directed at the target.

As soon as the target and camera were in place, baseline colony activity (number of forager departures over 4 min) was recorded on video. Following this, the end of a 1.5 m transparent plastic tube (2 cm internal diameter) was placed into the nest entrance and one of the researchers exhaled into the opposite end three times (Jandt, Detoni et al. 2020). We continued recording for two minutes, after which we removed all the materials and moved to the next nest. All materials were cleaned in between trials. For consistency purposes, the same researcher did the exhaling in every trial.

Using J-watcher (version 1.0, jwatcher.ucla.edu), we quantified baseline activity and the amount of time colonies engaged in defence flight (post-disturbance) (see Chapter 3 for definition of colony-level behaviours). The duration of defence flight behaviour after disturbances was used as a proxy for colony aggressiveness.

4.3.3 Genetic Sample Collection and Preparation

Between the 16 and 18 of March 2019, we collected foragers and defenders from three different *V. vulgaris* colonies. While it was undisturbed, we collected five wasps as they were returning to the nest in a straight path typical to foragers (hereafter so named), and immediately placed them on dry ice. This way we avoided collecting individuals taking their first flight (orientation flight) or those exiting the nest in response to presence of the researcher. Wasps were collected in a net approximately 30-50 cm from the nest entrance. After foragers were collected, colonies were experimentally disturbed placing the end of a 1.5 m transparent plastic tube (2 cm internal diameter) into the nest entrance and having one researcher exhale into the opposite end three times (simulating the approach of a mammal) (Jandt, Detoni et al. 2020). Wasps exiting the nest at least 30 seconds after the disturbance and displaying the defence flight behaviour (hereafter "defenders") were caught in the net, five of which were immediately placed on dry ice. Wasps were stored at -80^{o}C.

For RNA extraction, wasp heads were separated from the bodies and freeze-dried (Zirbus Technology VaCo 2-II freeze drier). Brains were dissected on dry ice and then stored at -80^{o}C. Total RNA of individual forager/defender heads was extracted using a Qiagen RNeasy Mini Kit. Given the size of the head tissue (< 20mg), a modified protocol for the purification of total RNA from animal tissues (RNeasy Mini Handbook 4th ed.) was used, where heads were homogenized with 350µl of RLT Buffer (Qiagen). DNase digestion was performed on all samples to remove potential contamination of genomic DNA. RNA was eluted with 60µl of RNase free water. The concentration (ng/µl) and quality assessments, via 260/280 and 260/230 ratios, of RNA extracts were measured using a DS-11 Spectrophotometer (DeNovix). Eighteen extract samples were further purified using a New England Biolabs Monarch® RNA clean-up columns. Based on purification results, 4 out of 5 samples per colony were chosen for TruSeq stranded mRNA library construction and sequencing based on highest concentration yield to ensure adequate sample quality.

Samples were sequenced on two lanes of an ILLUMINA HiSeq 2500 machine with V4 chemistry to produce 125bp paired-end reads at the Otago Genomics facilities.

4.3.4 RNA Sequencing Analysis

The complete RNA-Seq analysis is available in the Appendix (Supplementary Methods 1 and 2). Reads were quality-controlled using FastQC v0.11.9 (Barbraham Bioinformatics 2011). Illumina universal adapters were removed from the sequences using Cutadapt v3.3 (Martin 2011). Reads were quantified against the *Vespula vulgaris* reference genome (Harrop, Guhlin et al. 2020) using HISAT2 v2.2.1 (Kim, Langmead et al. 2015). After index building, reads were mapped using HISAT2 paired-end default parameters. Alignment files were sorted and compressed using Samtools v1.12 (Li, Handsaker et al. 2009). Finally, read counts per gene per sample were obtained using featureCounts v1.6.2 (Liao, Smyth et al. 2014).

For differential expression analysis, we used a pipeline based on the DESeq2 package v1.22.2 (Love, Huber et al. 2014) implemented in R (R Core Team, 2020, version 4.0.4). After filtering out genes with low statistical power using the *filterByExpr()* function of edgeR v3.24.3 (Chen, Lun et al. 2016), we assessed sample clustering using a Principal Component Analysis as implemented in *PlotPCA()* of DESeq2. First, we performed an analysis contrasting defenders and foragers accounting for colony; due to limitations in the statistical power of this analysis, we chose a p-value cut-off after adjusting for multiple testing (q-value) of 0.1 (Benjamini and Hochberg 1995). We then performed three additional separate analyses contrasting castes within each colony with the same significance threshold. Differentially expressed genes annotation was sourced from Harrop, Guhlin et al. (2020).

4.4 RESULTS

4.4.1 Colony Phenotypes

By looking at the duration of defence flight behaviour as a proxy for colony aggression phenotypes, all three colonies exhibited observable differences in aggressive response to the experimental disturbance across the 3 field trials (Fig. 4.1). Colonies L and M were consistently aggressive throughout trials. Overall, the aggressive response of colony L lasted

for 118.3±2.9 seconds (mean ± SD), and colony M showed maximum recorded defence duration (120s) in all 3 trials. Colony N showed the most variable response (58±25s), with nest defence effort declining over trials.

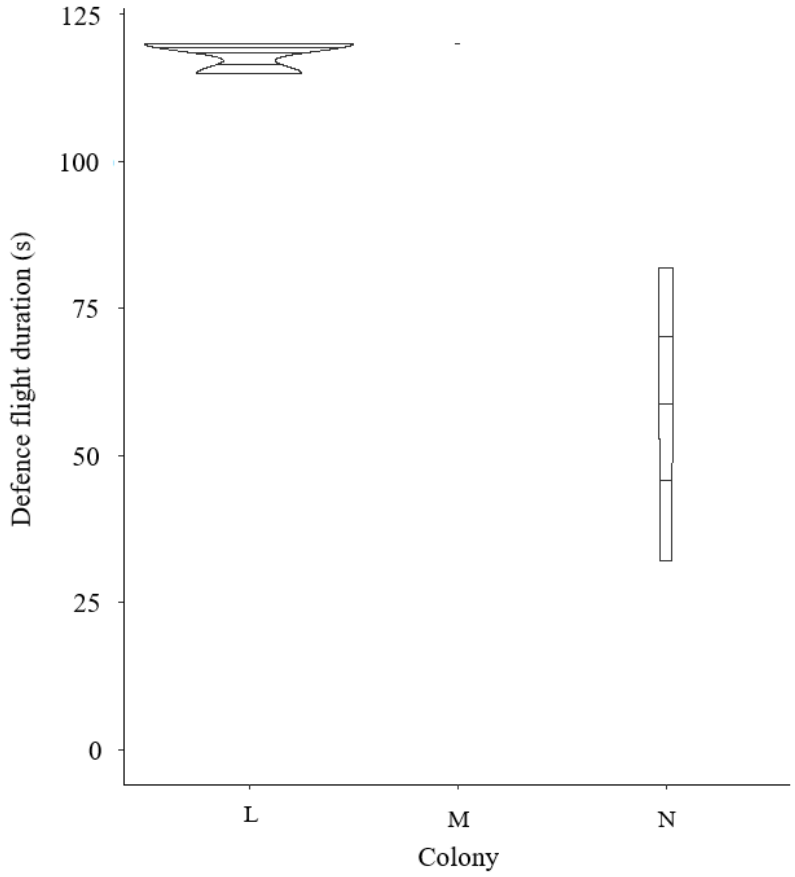

Figure 4.1. Collective aggression behaviour of *Vespula vulgaris* defenders (measured in defence flight duration) in three colonies (L, M, and N) over three trials over three weeks, March/April 2019. Horizontal lines within violin plots represent quartile and median distribution of defence flight duration, while plot width represents frequency density. Colony M showed maximum defence flight duration in all three trials.

4.4.2 RNA Sequencing Data

We sequenced 24 samples with 2 x 125bp paired-end reads producing a total of 401.1 million reads (range: 13.8 – 18.2 million reads per sample). After adapter trimming, mapping and quantification, we obtained 222.8 million reads (range: 8.2 - 10.6 million reads per sample). When contrasted to the whole-body reference transcriptome of *V. vulgaris* (Harrop,

Guhlin et al. 2020), 12,843 out of 15,512 genes (82.8%) in the annotation are found to be expressed in the brain.

4.4.3 Foragers versus Defenders: Brain Gene Expression

After filtering for low-expression genes to maximize statistical power (Chen, Lun et al. 2016), 9,025 genes and >99.9% reads were preserved. Principal component analysis (PCA) of samples showed no visible clustering according to behavioural caste (Fig. 4.2). Moderate clustering of samples could be observed for two of the three colonies, with samples collected from colonies L and M clustering together along the PC1-axis, in contrast to samples from colony N.

When contrasting castes correcting for colony effects, we identified three differentially expressed genes (DEGs) ($q < 0.1$; Table 4.1; Fig 4.3). HZH66_000687 was up-regulated in defenders and down-regulated in foragers (Fig. 4.3: HZH66_000687). Both HZH66_006027 and HZH66_007921 were down-regulated in defenders and up-regulated in foragers (Fig. 4.3: HZH66_006027 and HZH66_007921, respectively).

Further contrasting castes within each colony separately returned 19 additional genes, one which was found in colony N (HZH66_001901; Fig. 4.4B), and 18 of which were found within colony M (Fig 4.4A , Table 4.1). There was no overlap between DEGs identified within or across colonies, nor between colonies for the 19 genes found to differ between castes within colonies.

4.4.4 Differences Among Colonies: Gene Expression

Despite the low number of DEGs between behavioural castes, contrast among colonies revealed significant differences in brain gene expression (Fig. 4.5). Pairwise comparisons showed 3,097 DEGs (34.97% of genes; Fig. 4.5A) between colonies L and M, 5,267 DEGs (58.82% of genes; Fig. 4.5B) between colonies L and N, and 2,663 DEGs (29.77% of genes; Fig. 4.5C) between colonies M and N.

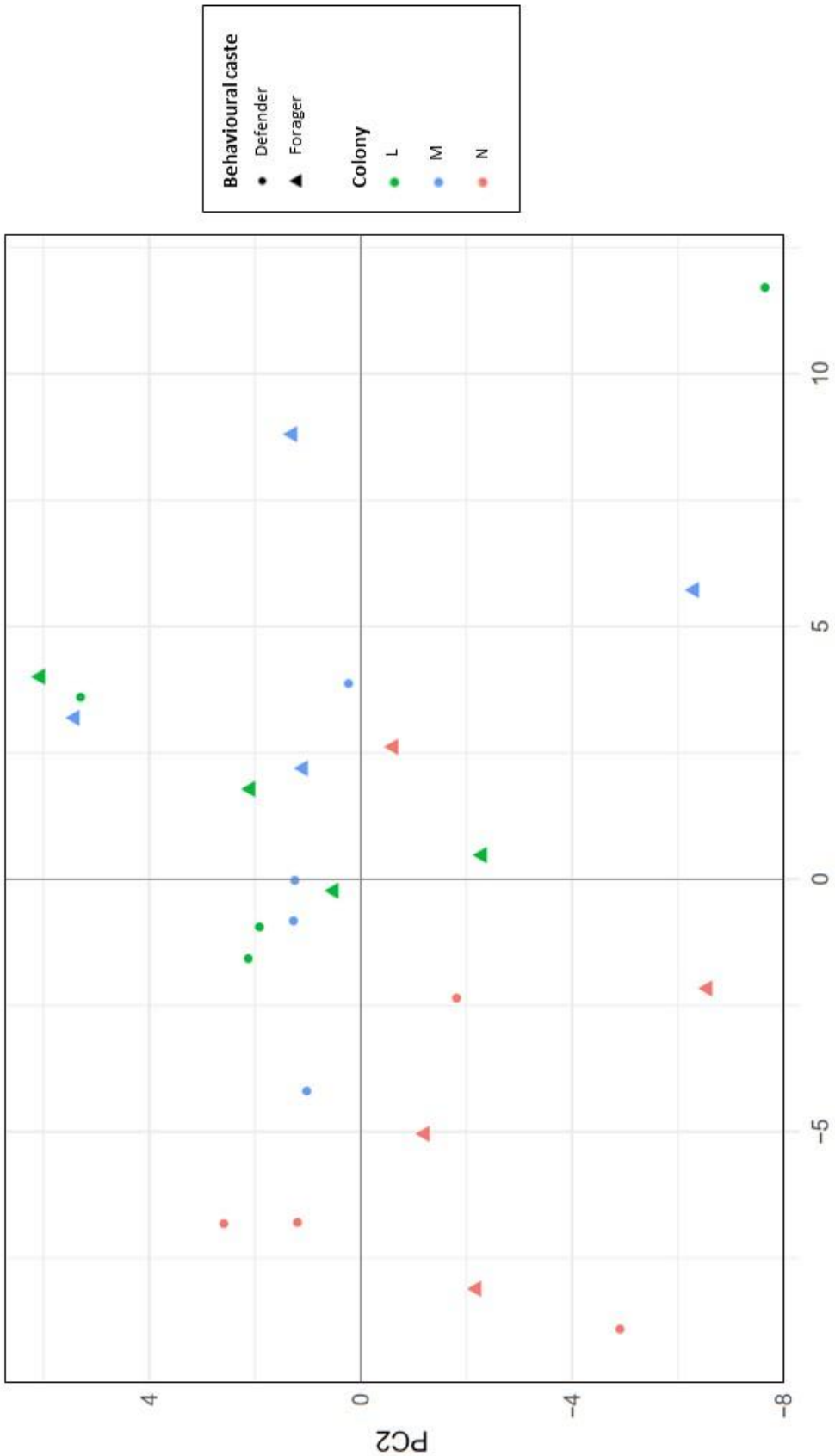

Figure 4.2. Principal component analysis (PCA) of whole-sample expression profiles between foragers and defenders of *Vespula vulgaris*, measured from brain RNA extractions. Samples are coloured by colony. Defender samples are shown in round markers, forager samples are shown in triangle markers.

Table 4.1. Differentially expressed genes between foragers and defenders of *Vespula vulgaris* measured from brain RNA extractions ($q < 0.1$). Significantly expressed genes presented according to whether defenders and foragers were contrasted across all colonies or within a specific colony (L, M, or N). The predicted molecular function is included for each gene.

Caste differences across colonies			
Gene name	**Log2-fold change**	**Contrast**	**Molecular function**
HZH66_000687	-0.234	All	Leucine rich adaptor
HZH66_006027	0.168	All	Unknown
HZH66_007921	0.173	All	Zinc finger
Caste differences within colonies			
Gene name	**Log2-fold change**	**Contrast**	**Molecular function**
HZH66_001901	1.173	N	Solute carrier family 12
HZH66_001767	-1.188	M	CLASP N terminal
HZH66_001973	-0.576	M	Protein kinase C conserved region 2 (CalB)
HZH66_003517	0.626	M	Ligand binding domain of hormone receptors
HZH66_003521	0.863	M	Ligand binding domain of hormone receptors
HZH66_003696	1.344	M	Peptidase family M13
HZH66_004750	0.431	M	ZnF_C4 abd HLH domain containing kinases domain
HZH66_005622	0.420	M	Cytochrome P450
HZH66_005721	0.675	M	Unknown
HZH66_009409	0.714	M	Cytoskeletal protein binding
HZH66_009726	-0.716	M	Ras family
HZH66_010198	0.434	M	Beta-galactosidase
HZH66_012299	0.825	M	D-isomer specific 2-hydroxyacid dehydrogenase, 2c catalytic domain
HZH66_013150	-0.736	M	Unknown
HZH66_013364	0.655	M	Unknown
HZH66_014258	0.906	M	Actin filament binding
HZH66_014482	1.219	M	CD36 family
HZH66_014920	-0.546	M	Pyridoxal-dependent decarboxylase conserved domain
HZH66_014938	1.009	M	Domain present in PSD-95, Dlg, and ZO-1/2

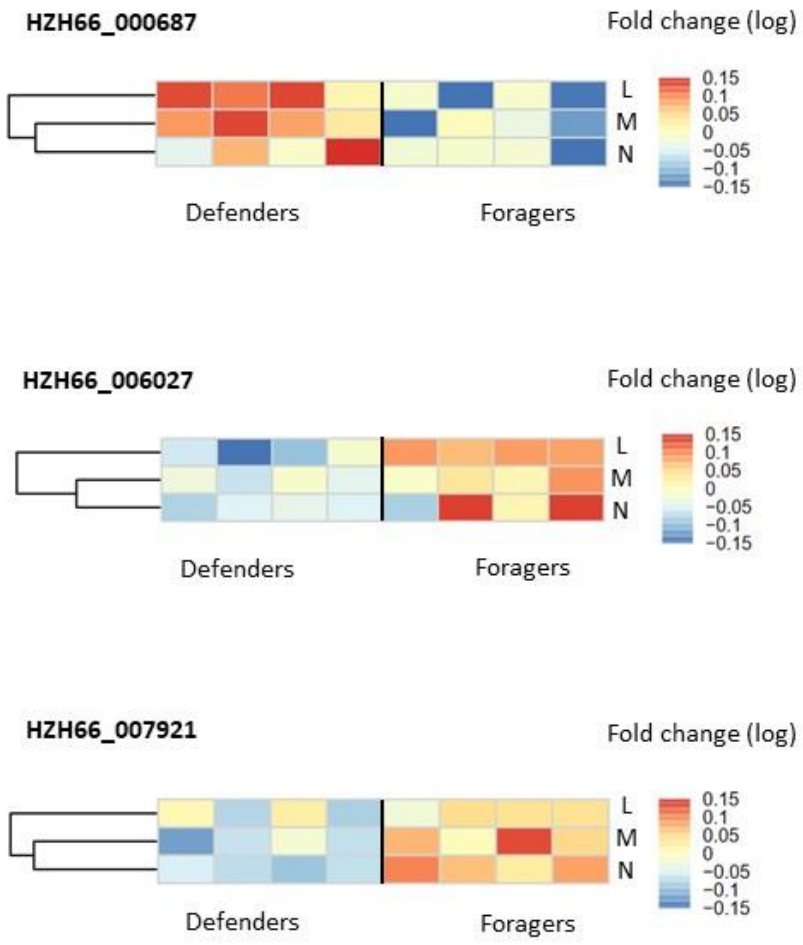

Figure 4.3. Heat maps of the scaled sample read counts for DEGs between foragers and defenders across all sampled colonies of *Vespula vulgaris*, measured from brain RNA extractions. Samples were obtained from three different colonies between 23/03 and 14/04/2019. Defender reads are grouped to the left of each map, while forager reads are grouped to the right. Log-transformed fold change rates shown in a blue scale when down-regulated, red scale when up-regulated, and yellow scale when unchanged.

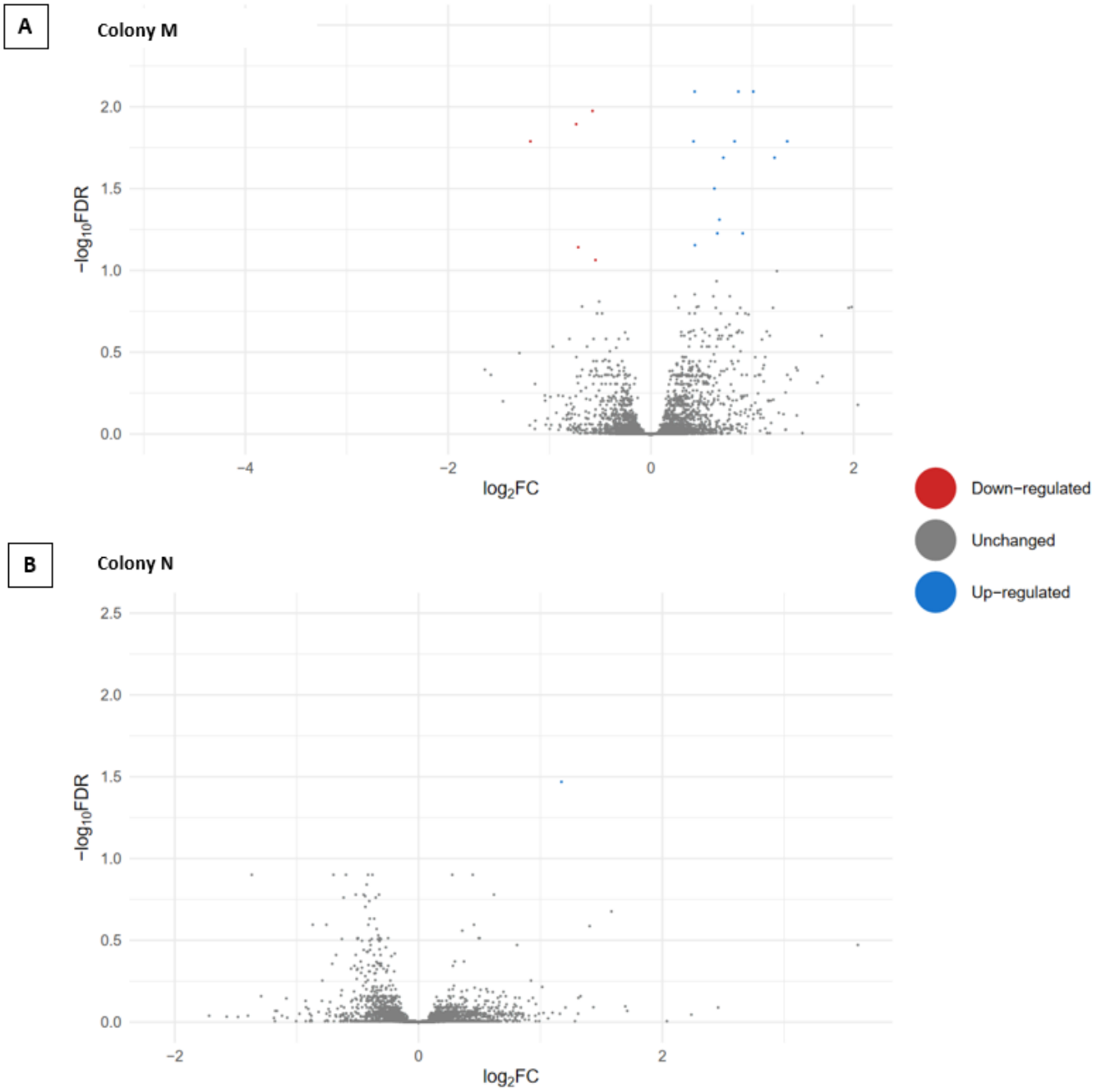

Figure 4.4. Volcano plots of DEGs found in defenders within *Vespula vulgaris* colonies, measured from brain RNA extract. **A**: contrast within colony M. **B:** Contrast within colony N. Points represents expression of genes in the *V. vulgaris* genome. The y-axis of each plot shows the -log10 p-value for each transcript based on the statistical analysis of pairwise colony comparisons. The x-axis of each plot shows the log2-fold change of each transcript. Significantly differentially expressed transcripts are shown in red (down-regulated) and blue (up-regulated). Non-significant DEGs are shown in grey. Colony M showed 5 up-regulated genes, 8 down-regulated genes, and 8,847 unchanged genes in defenders, while colony N showed 1 down-regulated gene, and 8,845 unchanged genes.

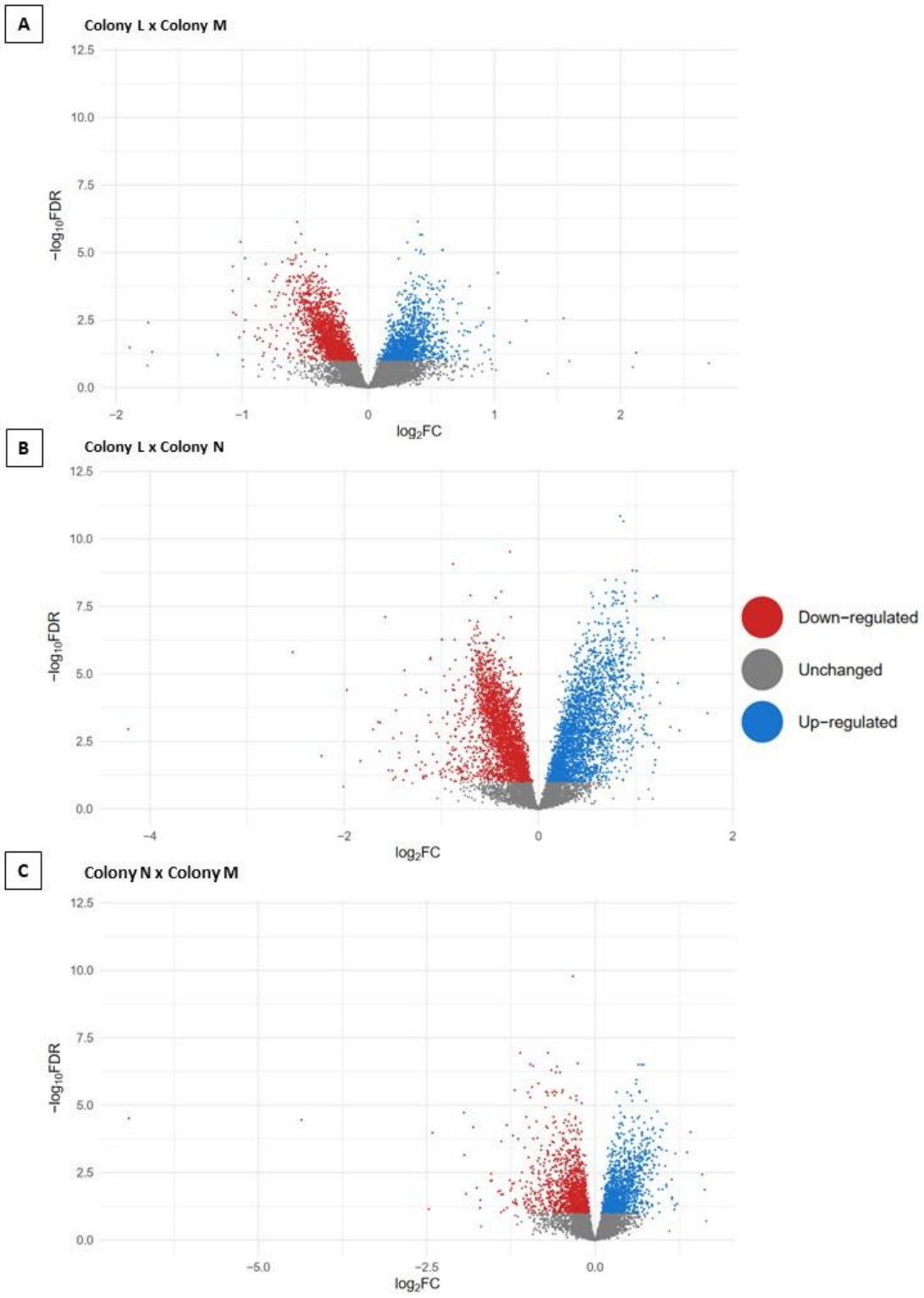

Figure 4.5. Volcano plots of pairwise comparisons of DEGs found in defenders between *Vespula vulgaris* colonies, measured from brain RNA extract. **A:** Contrast between colonies L and M. **B:** Contrast between colonies L and N. **C:** Contrast between colonies M and N. Points represents expression of genes in the *V.*

vulgaris genome. The y-axis shows the -log10 p-value for each transcript based on the statistical analysis of pairwise colony comparisons. The x-axis shows the log2-fold change of each transcript. Significantly DEGs are shown in red (down-regulated) and blue (up-regulated). Non-significant DEGs are shown in grey. Colony L showed 1,751 up-regulated genes, 1,346 down-regulated genes, and 5,857 unchanged genes when contrasted with colony M, while showing 2,693 up-regulated genes, 2,574 down-regulated genes, and 3,687 unchanged genes when contrasted with colony N. Colony M showed 1,331 up-regulated genes, 1,332 down-regulated genes, and 6,291 unchanged genes when contrasted with colony N.

4.5 DISCUSSION

In this chapter, we compare the brain transcriptomes of foragers and defenders across three colonies of *Vespula vulgaris*, to identify differentially expressed genes that might be linked to aggression behaviours in a nest defence context. The contrast between foragers and defenders revealed a small number of DEGs between foraging and defensive castes, both within some colonies and across all samples. Conversely, colonies strongly differed among each other in their workers' brain gene expression profiles, in addition to showing differing aggression behavioural phenotypes.

The differential expression analysis contrasting foragers to defenders of *V. vulgaris* revealed only three differentially expressed genes in the brain. These results contrast to those found in investigations comparing honey bee foragers and defenders of similar lineages (Alaux, Sinha et al. 2009), which identified between 29 and 97 DEGs. A possible explanation for this discrepancy between present results and those found by Aulax, Sinha et al. (2009) is that honey bees not only have a more pronounced division of labour system (Breed, Robinson et al. 1990, Robinson 1992), but also the higher levels of genetic diversity than that in *Vespula* due to the existence of more patrilineal lines ("superpolyandry"; Withrow and Tarpy 2018).

Our results suggest that the differentiation of *Vespula* workers into different out-of-nest behavioural castes has a weak transcriptomic basis – there are two possible explanations. Firstly, both foragers and defenders (and generally, workers who perform out-of-nest tasks) typically belong to older age groups in social insects (Wilson 1982, Breed, Robinson et al. 1990, Hurd, Jeanne et al. 2007). The transition from in to out-of-nest tasks as individuals age has been shown to be mediated by shifts in gene expression patterns (Sumner, Pereboom et al. 2005, Berens, Hunt et al. 2015). Thus, gene expression patterns may be more important

in the differentiation of in- and out-of-nest tasks, rather than the differentiation of tasks outside of the nest. This is further reinforced by the fact that genetic mechanisms are highly conserved across insect and even vertebrate taxa (Toth, Tooker et al. 2014), and thus aggression-dedicated individuals may share transcriptomic pathways with other behavioural groups, despite phenotypical differences (Rittschof and Grozinger 2021). Secondly, looking into other molecular mechanisms (e.g. at the proteomic or physiological scale) may explain the ontogeny of consistent individual differences in insects that lack obvious genotypic or transcriptomic differences across behavioural castes (Pinter-Wollman 2012, Rittschof and Grozinger 2021). A way to overcome the challenge posed by shared transcriptomic pathways in out-of-nest worker castes would be to investigate the contrast of brain gene expression patterns across cohorts of known age workers.

When contrasting foragers and defenders within each colony individually, we found 18 DEGs in colony M alone. While colony M had the most aggressive phenotype of all colonies, it remains comparable to colony L within the constraints of the behavioural observation methods used. Furthermore, colony M showed a comparable number of reads to other colonies, making it unlikely that this result is due to statistical power issues alone.

The small number of genes of interest found in this study make accurate gene ontology analysis unfeasible, and limits possible inferences on the mechanisms in which these genes may act to modulate aggression behaviour. Such limitations are expected when analysing non-model organism data (Ferreira, Patalano et al. 2013, Berens, Hunt et al. 2015). Still, some insight can be gained from the literature based on two out of three DEGs found to significantly differ between foragers and defenders across the whole sample, to which a predicted molecular function could be assigned. Up-regulated zinc finger encoding genes have also been linked to defensive (maternal) aggression and aggressive stress response in mice (Gammie, Auger et al. 2006, Malki, Tosto et al. 2016), while a family of zinc finger genes (*fruitless*) regulate social aggression in *Drosophila* fruit flies (Vrontou, Nilsen et al. 2006). These comparisons with our results suggest that zinc finger genes may be a common mechanism modulating animal aggression at the molecular level across taxa. On the other hand, the down-regulation of leucine-rich adaptor protein genes seen in defenders is likely related to foraging behaviour, rather than aggression. Leucine-rich adaptor protein genes

have been observed to be up-regulated in cattle with higher feeding efficiency (Mukibi, Vinsky et al. 2018).

Studies comparing social wasp workers to reproductive castes revealed more observable differences at the transcriptomic level than those shown here. For instance, Geffre, Liu et al. (2017) showed 51 differentially expressed transcripts (DETs) when contrasting healthy *Polistes dominula* (Vespidae: Polistinae) workers to those infected with *Xenos vesparum*, which show gyne-like behavioural patterns (Geffre, Liu et al. 2017). The contrast is even higher when workers and actual reproductive castes are compared: 305 DETs in *P. dominula* (Geffre, Liu et al. 2017) and 736 DETs in *Polistes metricus* (Berens, Hunt et al. 2015). This discrepancy between the transcriptomic patterns described in those studies and here are not surprising, given that reproductive castes typically have significantly different life histories than workers and display a distinct range of behaviours (Spradbery 1973). Furthermore, the small amount of DEGs found here are unlikely to be a by-product of methodological limitations or lack of statistical power in the analysis. This is evidenced by the clear differences in gene expression patterns identified between, rather than within, colonies. Hence, although the experimental design could benefit from a higher sampling effort, our results still reflect significant biological processes underlying caste differentiation in *Vespula*.

Across-colony differences in aggression are shown here at the phenotype (behaviour) and molecular (transcriptome) level. Defensive aggression is a useful model for the study of behavioural consistency in social insects, since aggression displays are often clearly observable (Archer 1998), and anti-predator efforts often show consistent variation across individuals, behavioural castes, and colonies in social insects (Hölldobler 1983, Breed, Robinson et al. 1990, DeGrandi-Hoffman, Collins et al. 1998, Scharf, Modlmeier et al. 2012, Santoro, Hartley et al. 2015, Jandt, Detoni et al. 2020). *Vespula* are no exception when it comes to aggressive behaviour: colonies have been shown to vary consistently in their predator response both within and across species (Jandt, Detoni et al. 2020; also see Chapter 3). These consistent differences in colony aggressiveness can be at least partially explained by hereditary molecular factors. In honey bees (*Apis mellifera*), for instance, African lineages tend to be consistently more aggressive than their European counterparts (DeGrandi-Hoffman, Collins et al. 1998), even when cross-fostered by each other (Alaux, Sinha et al.

2009), and similar differences in aggression-related transcriptomic patterns between lineages have been demonstrated (Alaux, Sinha et al. 2009). The pronounced differences between *V. vulgaris* colonies shown here are thus expected, not just because there was phenotypical variation in aggression behaviours, but also due to the variation in genotypes intrinsic to each colony. *Vespula* usually have a low genetic diversity within colonies, with all workers typically sharing the same mother and an average 2-3 patrilineal groups (Goodisman, Matthews et al. 2002). In this sense, genomic differences should be present at the population level in a much higher magnitude than at the colony level. Future investigations of population genetics, including greater colony samples, could help clarify whether there are heritability mechanisms underlying the consistent aggression variation found between colonies.

Overall, we found limited evidence of a transcriptomic base for caste differentiation between foragers and defenders in *Vespula* social wasps, and generated a brain transcriptome of *V. vulgaris* for the first time. Our result agrees with a relatively weak division of labour and the fact that the two castes often share age groups (Hurd, Jeanne et al. 2007, Santoro, Hartley et al. 2015), which possibly results from the development of associated genomic pathways between them (Toth, Tooker et al. 2014, Rittschof and Grozinger 2021). We also show evidence to support strong gene expression differences across colonies which, adding to existing evidence on consistent behavioural differences across colonies (Jandt, Detoni et al. 2020), can help guide future investigations on the link between behaviour and genetics at the population level.

Chapter Five

General Discussion and Final Thoughts

Social insects are good candidates to study aggression, due to: (1) being easily observable in controlled and natural settings (Archer 1998, Rittschof and Grozinger 2021); (2) showing significant variation within and across colonies (Jandt, Bengston et al. 2014, Jandt, Detoni et al. 2020); and (3) being influenced by a range of different biological mechanisms, from the macroecological (e.g. abiotic and social; Southwick and Mortitz 1987, Santoro, Hartley et al. 2015) to the developmental (e.g. molecular; Smith, Toth et al. 2008, Avalos et al. 2020) level. In this thesis, I investigated the defensive aggression behaviour of the social wasp *Vespula vulgaris* in the context of nest defence, and quantified the role of different ecological and molecular influences on behavioural variation at the individual and colony levels.

5.1 Contributions to the Fields of Behavioural Ecology and Sociobiology

Here, I explored different mechanisms underlying the variation of aggression behaviours in social wasps. I synthesised predatory events on social wasps around the globe and analysed emerging biogeographic variation patterns. In Chapter 2, I showed that Carnivora mammals (namely bears, badgers, skunks) and other social Hymenoptera (especially army ants and hornets) are the main predators of social wasps, none of which are present in New Zealand. Where introduced social vespids cause major ecological imbalance (MacIntyre and Hellstrom 2015, Lester and Beggs 2019), the absence of predators can help explain their colonisation success (Godfrey 1995, Barlow, Beggs et al. 2002). The ecological relationships between wasps and their predators can be used to better understand predatory pressures (or lack thereof) faced by introduced wasp populations in non-native ranges. In contrast, it can also support investigations into wasp interactions in native habitats. Considering the recent debate on major worldwide shifts in insect biodiversity (Hallmann et al. 2017), understanding how predator diversity might indirectly impact wasp populations can be a key aspect to consider when monitoring the group's conservation. My findings add to the evolutionary history of wasps regarding predator pressure (Starr 1985, Hunt 2007,

Schmidt 2016), strengthening the importance of defensive behaviours for the evolution of eusociality (Rittschof and Grozinger 2021). Finally, a first ever comprehensive list of predators for social Vespidae worldwide is provided, both at the individual and at the colony level, adding to the present knowledge on the life history of wasps, which so far have relied mostly on anecdotal evidence and general statements (West-Eberhard 1969, Spradbery 1973, Ross and Matthews 1991).

In Chapter 3, the likely absence of native specialised predators of *Vespula* colonies in New Zealand meant that I could act as the sole disturbance to determine how development or experience affect colony-level variation of nest defence aggressiveness in *V. vulgaris*. The most reliable predictor of aggression found in my experiments is the effect of the colony cycle. The role of seasonality and the pacing of the colony cycle agree with previous investigations in the related species (Judd 1998, London and Jeanne 2003) and supports previously reported high synchronicity of life histories observed in temperate species of the group (Ross and Matthews 1991). Considering social life evolution, I show an indirect relationship between a colony's changing demography (brood:worker ratio) and its behavioural interactions with the environment – a relationship predicted by the "superorganism" thesis (Hölldobler and Wilson 2008). Furthermore, I indicate the limited short-term plasticity and high individual variability of aggression behaviours at the colony level, also seen in previous studies (Jandt, Bengston et al. 2014, Wright et al. 2019, Jandt, Detoni et al. 2020). My results, in conjunction with previous studies, suggest nest defence behaviours are a good model for future investigations on the ecological and evolutionary significance of consistent behavioural variation in the social Hymenoptera.

Finally, in Chapter 4, I studied the association between transcriptomics in *V. vulgaris* aggression at the individual level by comparing brain gene expression between nest defenders and foragers within and across colonies. I provided evidence that the differentiation between out-of-nest behavioural castes have weak transcriptomic bases in *Vespula*, which is in line with the overall weak division of labour system described for vespine wasps (Hurd, Jeanne et al. 2007, Santoro et al. 2019). Regarding the study of defensive aggression, and under the light of temporal polyethism, I suggest that (from an omics/developmental perspective) nest defence is likely undertaken generally by older worker cohorts within the colony, rather than a discrete dedicated group of guards (Santoro, Hartley et al. 2019). Because behavioural

castes are a staple of eusocial life in the Hymenoptera (Gordon 2016), these findings contribute to the understanding of the evolution of division of labour systems, which evolved differentially in wasps when compared to ants or bees (Robinson 1992, Gordon 1995). The results described in Chapter 4 add to those found in Chapter 3 by supporting a strong base for inter-colony phenotypical and molecular variation linked to aggression behaviours. These arguments inspire future investigations on the ontogeny and fitness of colony behaviours in wasps, which may be supported by the brain transcriptome I make available for *V. vulgaris*.

5.2 How do Defensive Aggression Phenotypes Arise in *Vespula vulgaris*?

The main goal of this thesis was to examine different biological mechanisms underlying the variation of aggression phenotypes in a social wasp. Behavioural phenotypes arise from an incredibly complex interaction of developmental and ecological factors (Foster 2013), making this question impossible to answer fully based only in a limited number of investigations, even when only a single system is considered. When combined, my results make up a piece of that puzzle by explaining some of the variability observed in *Vespula* aggression (Jandt, Detoni et al. 2020).

Plenty of published evidence supports the claim that ergonomic colonies – those who are at the peak of their activity and show high brood:worker ratios – are more prone to react aggressively to predator disturbances (Gordon 1991, Gordon 1995, Judd 1998, Dreller and Tarpy 1999, London and Jeanne 2003, Wray, Mattila et al. 2011). By considering the synchronicity of *Vespula* colony cycles in temperate areas (Greene 1991, Barlow, Beggs et al. 2002), we can also infer that abiotic factors have an indirect influence on aggression over long periods of time – for example, the higher environmental temperatures that often coincide with the ergonomic phase of the cycle (Huffaker and Gutierrez 1999). However, I found no evidence to support a direct effect of microvariation in abiotic variables on aggressive phenotypes over shorter terms, which differs from what has been described in other social insect groups (Southwick and Mortitz 1987, Segev, Burkert et al. 2017, Horna-Lowell, Nuemann et al. 2021). Additionally, despite sensitization being described for individual worker of *Vespula* colonies (Santoro, Hartley et al. 2015), it is possibly not a relevant

mechanism underlying nest defence at the colony level, at least for New Zealand populations of the genus (London and Jeanne 2003).

The transcriptomic similarities between out-of-nest workers shown here agree with the temporal polyethism suggested to underlie task division in the group (Hurd, Jeanne et al. 2007), but also in other Hymenoptera societies (Hölldobler and Wilson 1990, Giray, Guzmán-Novoa et al. 2000, O'Donnell 2001). Although I did not specifically address the effect of behavioural consistency at the colony level in my main questions, the existence of strong phenotypical and transcriptomic variation across colonies was a common finding in my experiments. While my thesis does not provide a clear answer for the ontogeny of this variability, I suggest that looking at mechanisms not addressed here – especially intrinsic ones such as physiological and genomic (Bengston and Jandt 2014, Rittschof and Grozinger 2021) – may be the main route to understand how aggressive phenotypes arise in social wasps.

5.3 Challenges and Limitations

Field-based investigations in behavioural ecology are useful for the understanding of the ecological and evolutionary significance of animal behaviour in their natural context, avoiding much of the bias created in artificial systems (e.g. Jandt, Thomson et al. 2015). However, studying organisms *in natura* adds to the already complex interaction of factors that may or may not influence observed behaviours, and makes the isolation of single causal mechanisms especially challenging. Large sample sizes (especially at the colony level, for social wasps; e.g. Nandi, Sumana et al. 2014, Brito, Aragão et al. 2018, Jandt, Detoni et al. 2020) are not always readily available, constraining statistical modelling power. Adding to these challenges, the process of experimental design is further limited by (1) relying on ideal weather conditions to allow for the observation of out-of-nest behaviours, as well as standardizing data collection, and (2) accommodating for the limited availability of colonies due to the seasonality aspect of their life histories in New Zealand (Barlow, Breed et al. 2002).

In addition to the constraints intrinsic to field experiments, other challenges arose during the development of my studies. Originally, I planned to integrate more controlled

experiments to my thesis by rearing wasps in semi-artificial containment. By combining the results found in the field to those in more refined experimental settings, I expected to provide a clearer answer to what mechanisms influence the determination of aggressive phenotypes in *V. vulgaris*. Although relatively cheap methods for rearing *Vespula* in semi-artificial containment are described in the literature (Jandt and Jeanne 2005, Jandt, Taylor et al. 2010), these still require significant infrastructure to be feasible (i.e. a dedicated outdoor site where wasps are able to freely forage). Due to the logistics involved in acquiring such a structure, and because social wasps suffer from a significantly negative perception from the general public (Sumner, Law et al. 2018), it was not until 2020 when I was able to secure the necessary resources to set controlled experiments up. Unfortunately, the work restrictions imposed by the global COVID-19 pandemic forced me to shut down experiments during the season when wasps are active (Barlow, Breed et al. 2002), and thus only previously collected field-based experimental data could be included in this thesis.

Finally, a challenge I faced when discussing my results has to do with the invasion ecology of *Vespula* in New Zealand. It has recently been shown that the introduction of *Vespula* involved a significant genetic bottleneck effect (including for *V. vulgaris*; Schmack, Brenton-Rule et al. 2019). Assuming a strong relationship between genetic and behavioural diversity, which is supported by the intrinsic aspect to the variation in aggression suggested by my results, this could mean that some of my conclusions are not widely applicable for the species or the genus in other areas, especially in their native range. In this regard, future investigations comparing behavioural variation across ranges could help validate the results I show here.

5.4 Future Perspectives

The findings shown here open a wide horizon for further investigating the subject. Most studies on underlying mechanisms of behavioural aggression in Hymenoptera focus on only a few taxa within bees (*Apis mellifera*, e.g. Collins, Rinderer et al. 1980, Breed, Guzmnán-Novoa et al. 2004, Alaux, Sinha et al. 2009), ants (e.g., van Wilgenburg, Clémencet et al. 2010, Modlmeier and Foitzik 2011, Wittman, O'Dowd et al. 2018), and wasps (the genus *Polistes*, e.g. Judd 1998, London and Jeanne 2003, Brito, Aragão et al.

2018). Here, I show the potential for *Vespula* to be added to that limited taxonomic list, considering its availability in areas such as New Zealand and the feasibility in observing its varying aggressive phenotypes.

One of the results outlined by Chapter 2 is the relatively small existing number of empirical studies dedicated to observe and describe wasp predation at the colony level in a quantitatively relevant way. To further understand the life histories of social wasps as prey, monitoring natural wasp populations through methods used for macrofauna conservation (e.g. motion activated cameras, Kelly 2008) through long periods would be an effective, if laborious, route. Another way to approach this gap in knowledge would be to use the predator list I provide to acquire further data in understudied areas, such as Oceania and Africa. By limiting the scope of investigations to known predator groups, indirect methods for evidencing predation (such as the analysis of stool samples; Birkhead 1974) could be a more feasible alternative to direct observations.

The experimental design described in Chapter 3 had logistic challenges that limited how frequently colonies could be disturbed. To address this, future research could be done in more controlled environments. Even with smaller sample sizes, a semi-artificial setting could allow for a greater manipulation of colonies and for simulated threats to be provided at a much more contrasting rate between treatments, picking up on potential experience-induced effects overlooked in my original design. Because experience at the colony level can be assumed to be mediated by social interactions between workers, these could also be observed and quantified in a containment setting. Drivers behind strong inter-colony variation in defensive aggression efforts outlined in Chapter 3 are yet to be identified. This variation seems to be strongly intrinsic to each colony, which could be confirmed by carrying out cross-rearing experiments between colonies of opposite aggressive phenotypes (DeGrandi-Hoffman, Collins et al. 1998). Contrasting the effects of "nature *versus* nurture" across colonies could provide valuable insights on which developmental factors underlie this diversity of aggressive types.

Because strong inter-colony variability of aggression was also found in Chapter 4 at the transcriptomic level, there are strong grounds for a link between genomics and nest defence behaviours in *Vespula*, even despite the low genetic diversity attributed to New Zealand wasp populations (Burne, Ritchie et al. 2017). A study on population genetics

comparing wasp colonies with various phenotypes could reveal the key factors behind the intrinsic mechanisms underlying phenotypical variation in nest defence. Identifying an "aggression gene" in *Vespula* would be an ideal, albeit ambitious, goal to pursue in future research. Furthermore, if a controlled experimental setting is established, comparing the brain transcriptome of in-nest to out-of-nest workers could add to my findings to support our discussion of shared transcriptomic pathways regulating cohort behaviours based on temporal transitions in their behavioural patterns (Hurd, Jeanne et al. 2007).

Finally, there are other factors that could help explain the arising of aggressive phenotypes in wasps that were not touched in this thesis. For instance, the effect of parasitism prevalence in colonies is suggested to play a major role in influencing nest defence behaviours (Phil Lester, personal communication). Knowing whether parasite-ridden colonies become more aggressive (as demonstrated for honey bees, Fujiyuki, Takeuchi et al. 2004, Lecoq, Jensen et al. 2016), or if the opposite is possible, would be a logical next step to address the questions I proposed to address here.

Other environmental elements somewhat tied to colony seasonality, and thus possibly affecting nest defence, include inter-colony competition for food resources (Downs and Ratnieks 2000, Wittman et al. 2018). Because New Zealand offers an extreme example of density of *Vespula* populations in some areas of the country (Barlow, Beggs et al. 2002), these settings are ideal for experiments. For instance, it could be interesting to further investigate if environments with super dense populations result in higher inter-colony competition for food, and whether there are shifts aggression to reflect that pressure.

5.5 Conclusion

My thesis provides solid evidence that aggression behaviours in *Vespula* wasps during nest defence arise in response to varied mechanisms. To fully understand how variation in this behaviour arises at the colony level, a strong understanding of the interaction of factors at different biological levels – from molecular to macroecological – is necessary. Although many investigations need to be carried out before a satisfactory explanation to that phenomenon can be provided, my work provides a glimpse to the final outcome by hinting

at the effects of predation pressure, colony seasonality, inter-colony behavioural variation, and intra-colony transcriptomic mechanisms at play.

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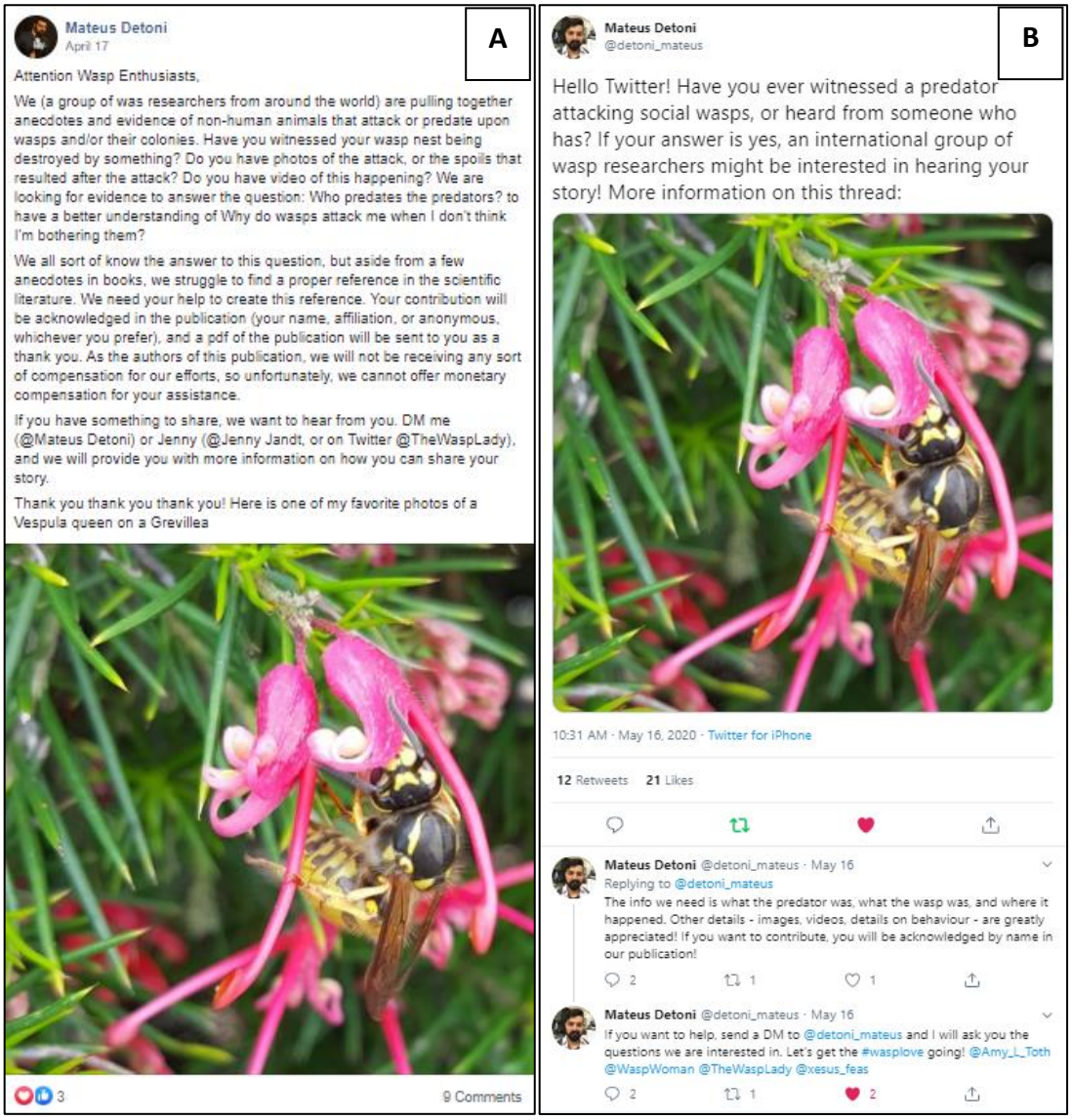
Appendix

Supplementary Figures



Supplementary Figure 1. Screen capture of messages sent in social media inviting users to share observation on social wasp predation with the authors. **A**: message posted by MD on Facebook groups dedicated to sharing of information on social wasps; **B**: thread posted publicly on Twitter by MD and publicly shared by JMJ and SS.

Attention Wasp Researchers,

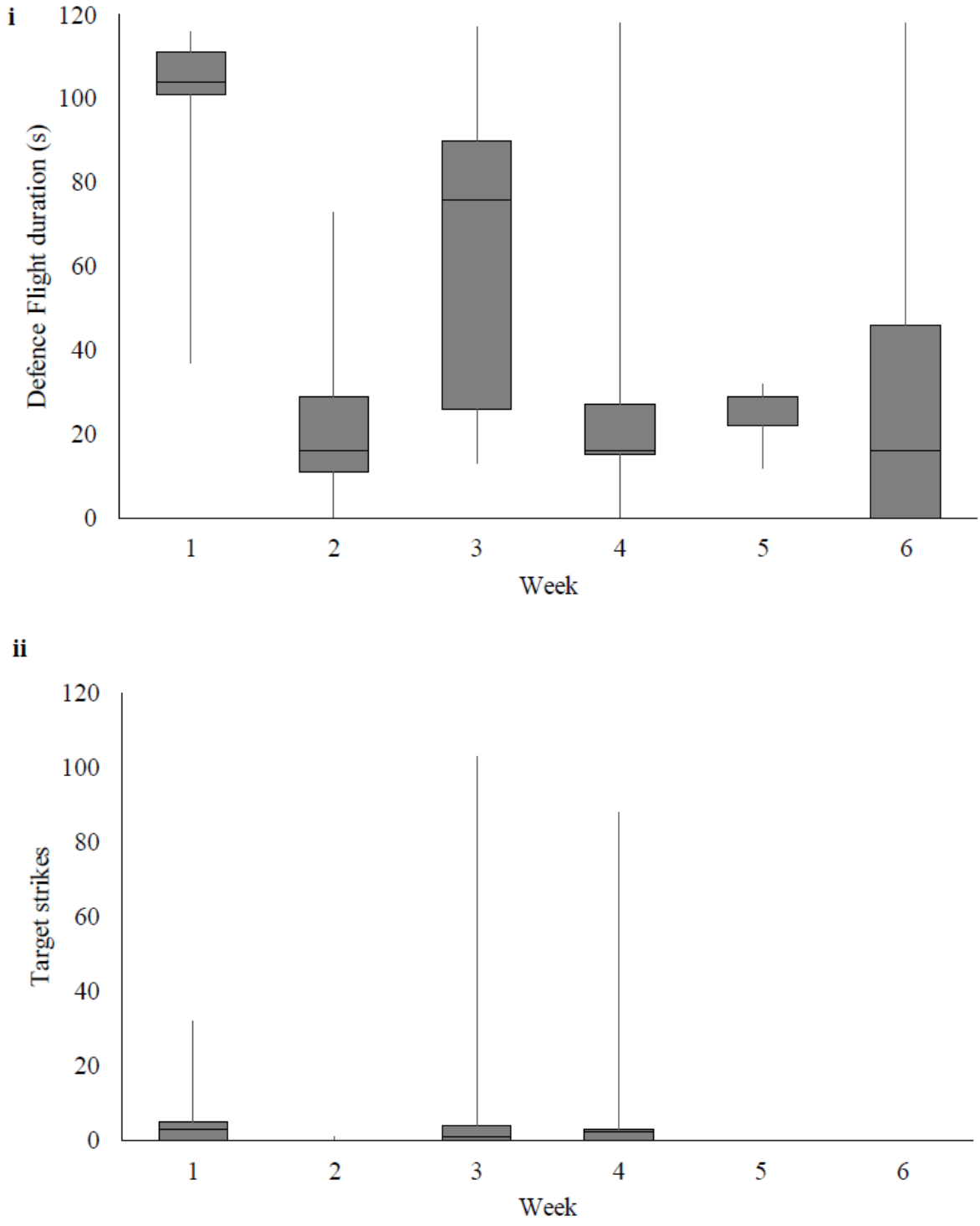
We (a group of was researchers from around the world) are pulling together anecdotes and evidence of non-human animals that attack or predate upon wasps and/or their colonies. Have you witnessed your wasp nest being destroyed by something? Do you have photos of the attack, or the spoils that resulted after the attack? Do you have video of this happening? We are looking for evidence to answer the question: Who predates the predators? to have a better understanding of Why do wasps attack me when I don't think I'm bothering them?

We all sort of know the answer to this question, but aside from a few anecdotes in books, we struggle to find a proper reference in the scientific literature. We need your help to create this reference. Your contribution will be acknowledged in the publication (your name, affiliation, or anonymous, whichever you prefer), and a pdf of the publication will be sent to you as a thank you. As the authors of this publication, we will not be receiving any sort of compensation for our efforts, so unfortunately, we cannot offer monetary compensation for your assistance.

If you have something to share, we want to hear from you. Send us an e-mail, and we will provide you with more information on how you can share your story.

Hope you are all well and healthy. Looking forward to hearing your feedback!

Supplementary Figure 2. Message sent via e-mail through the 'polistserv' listserv dedicated to information exchange between social wasp researchers throughout the world.



Supplementary Figure 3. Nest defence behaviors of *V. vulgaris* colonies in the 'hi-freq' treatment groups across all weeks. Weeks 2, 3, and 5 were not included in the data analysis. (i) Distribution of defence flight duration across weeks. (ii) Distribution of target strikes across weeks. Boxes represent quartile and median distribution of defence flight duration, lines, minimum and maximum values.

Appendix

Supplementary Methods

Supplementary Methods 1. Adapter trimming mapping and quantification analysis of RNAseq data from brain gene extracts of *V. vulgaris* foragers and defenders. Analysis code provided by Ludovic Dutoit.

[Methods attached separately]

Supplementary Methods 2. Differential expression analysis of RNAseq data from brain gene extracts of *V. vulgaris* foragers and defenders. Analysis code provided by Ludovic Dutoit.

[Methods attached separately]

Appendix

Supplementary Tables

Supplementary Table 1. Search terms used for literature search in different media. “General predation search” and “Prey-specific search” terms were used in the scientific literature and video (YouTube) searches, while “Predator-specific search” terms were used only in video searches (see Chapter 2, ‘Material and Methods’).

Search terms		
General predation search	Prey-specific search	Predator-specific search
"attack on social wasp nest"	"attack on Epiponini nest"	"ants vs wasp nest"
"attack on social wasps"	"attack on Epiponini"	"ants vs wasp"
"attack on Vespidae nest"	"attack on hornet nest"	"army ants vs wasp nest"
"attack on Vespidae"	"attack on hornets"	"army ants vs wasp"
"attack on wasp nest"	"attack on hover wasp nest"	"badger vs wasp nest"
"attack on wasps"	"attack on hover wasp"	"badger vs wasp"
"predator of social wasp nest"	"attack on Mischocyttarini nest"	"bear vs wasp nest"
"predator of social wasps"	"attack on Mischocyttarini"	"bear vs wasp"
"predator of Vespidae nest"	"attack on paper wasp nest"	"bird vs wasp nest"
"predator of Vespidae"	"attack on paper wasp"	"bird vs wasp"
"predator of wasp nest"	"attack on Polistinae nest"	"crow vs wasp nest"
"predator of wasps"	"attack on Polistinae"	"crow vs wasp"
	"attack on Polistini nest"	"hawk vs wasp nest"
	"attack on Polistini"	"hawk vs wasp"
	"attack on Ropalidiini nest"	"honey buzzard vs wasp nest"
	"attack on Ropalidiini"	"honey buzzard vs wasp"
	"attack on Stenogastrinae nest"	"hornets vs wasp nest"
	"attack on Stenogastrinae"	"hornets vs wasp"
	"attack on swarming wasp nest"	"human eating wasp nest"
	"attack on swarming wasp"	"human eating wasp"
	"attack on Vespinae nest"	"jay vs wasp nest"
	"attack on Vespinae"	"jay vs wasp"
	"attack on Vespini nest"	"magpie vs wasp nest"
	"attack on Vespini"	"magpie vs wasp"
	"attack on yellowjacket nest"	"monkey vs wasp nest"
	"attack on yellowjacket"	"monkey vs wasp"
	"predator of Epiponini nest"	"raccoon vs wasp nest"
	"predator of Epiponini"	"raccoon vs wasp"
	"predator of hornet nest"	"raven vs wasp nest"
	"predator of hornets"	"raven vs wasp"
	"predator of hover wasp nest"	"skunk vs wasp nest"
	"predator of hover wasp"	"skunk vs wasp"

Supplementary Table 1 (cont.)

"predator of Mischocyttarini nest"	"spider vs wasp nest"
"predator of Mischocyttarini"	"spider vs wasp"
"predator of paper wasp nest"	"wasps vs wasp"
"predator of paper wasp"	"weasel vs wasp nest"
"predator of Polistinae nest"	"weasel vs wasp"
"predator of Polistinae"	
"predator of Polistini nest"	
"predator of Polistini"	
"predator of Ropalidiini nest"	
"predator of Ropalidiini"	
"predator of Stenogastrinae nest"	
"predator of Stenogastrinae"	
"predator of swarming wasp nest"	
"predator of swarming wasp"	
"predator of Vespinae nest"	
"predator of Vespinae"	
"predator of Vespini nest"	
"predator of Vespini"	
"predator of yellowjacket nest"	
"predator of yellowjackets"	

Supplementary Table 2. Raw data file of all records of social wasp predation found in the literature search and included in this study, including details on predator and prey taxonomy, area and type of predation (individual vs. colony), medium, evidence type (including direct vs. indirect evidence classification), original observer and/or full literature reference (when appropriate). For video references, see Sup. Table 3.

[Table attached separately]

Supplementary Table 3. List of video records of social wasp predation found in the YouTube search. Video numbers are as cited in the main text and/or raw data file (Sup. Table 4), followed by URL (access on April-June 2020).

[Table attached separately]

Supplementary Table 4. Colonies of *V. vulgaris* at the Dunedin Botanic Garden used in nest defence trials.

Colony	Date found	Site	Frequency treatment	Date excavated
A	05/02/2018	Under bush	Hi-freq	14/05/2018
B	07/02/2018	Exposed	Hi-freq	14/05/2018
C	29/01/2018	Under bush	Hi-freq	-
D	08/02/2018	Under tree roots	Hi-freq	14/05/2018
E	12/02/2018	Exposed	Hi-freq	21/05/2018
F	09/01/2018	Under ivy	Lo-freq	14/05/2018
G	12/02/2018	Exposed	Lo-freq	14/05/2018
H	12/02/2018	Under ivy	Lo-freq	21/05/2018
I	12/02/2018	Under ivy	Lo-freq	-
J	12/02/2018	Under ivy	Lo-freq	-
K	05/02/2018	Amidst grass	Lo-freq	-

Supplementary Table 5. ANOVA outputs for the models used to identify significant predictor fixed effects of different response variables in our data. Bold values indicate that a covariate was a significant predictor of the response variable. F-ratio values between brackets indicate degrees of freedom ("numerator, denominator"). When multiple models are present for the same number (a, b), it indicates exclusion of non-significant interaction terms, with the last model (b) considered definitive.

Question	Response	Model	Factor/Covariate	Result	
Are the secondary covariates (foraging activity and abiotic factors) measured in the study correlated with each other?	Temperature	1 LMM	**Week**	**$F_{(2,30)} = 5.536$**	**$p = 0.009$**
	Humidity	2 LMM	Week	$F_{(2,30)} = 0.958$	$p = 0.395$
	Pre-disturbance foraging activity	3a GLMM	Week * Treatment	$F_{(2,17)} = 4.955$	$p = 0.040$[a]
		3b GLMM	**Week**	**$F_{(2,19)} = 51.94$**	**$p < 0.001$**
			Treatment	$F_{(1,9)} = 1.357$	$p = 0.274$
			Humidity	**$F_{(1,19)} = 7.656$**	**$p = 0.010$**
		4 GLMM	Treatment	$F_{(1,9)} = 1.355$	$p = 0.274$
			Temperature	**$F_{(1,21)} = 72.711$**	**$p < 0.001$**
Can nest defense behaviors be predicted by week, experience, or the secondary covariates?	Defense flight duration	5a LMM	Week * Treatment	$F_{(2,17)} = 0.901$	$p = 0.424$
		5b LMM	**Week**	**$F_{(2,19)} = 4.697$**	**$p = 0.022$**
			Treatment	$F_{(1,9)} = 0.109$	$p = 0.749$
			Humidity	$F_{(1,23)} = 1.504$	$p = 0.232$
		6 LMM	Pre-disturbance foraging activity	$F_{(1,27)} = 0.262$	$p = 0.613$
	Target strikes	7a GLMM	Week * Treatment	$F_{(2,17)} = 0.000$	$p = 1.000$
		7b GLMM	Week	$F_{(2,19)} = 3.022$	$p = 0.072$
			Treatment	$F_{(1,9)} = 2.066$	$p = 0.184$
			Humidity	$F_{(1,19)} = 0.829$	$p = 0.374$
		8 GLMM	**Pre-disturbance foraging activity**	**$F_{(1,21)} = 50.298$**	**$p < 0.001$**
Are the two behaviors used as a proxy for colony aggressiveness correlated?	Target strikes	9 GLMM	**Defense flight duration**	**$F_{(1,21)} = 50.759$**	**$p < 0.001$**

[a] p-value is non-significant after Bonferroni correction (conf. int. = 0.975)

Supplementary Table 6. Aggression phenotypes of *V. vulgaris* colonies, measured in duration of defence flight behaviour (s). Colonies showing the highest aggression in the first trial (bold) were chosen for genetic sample collection.

Colony	Defence flight duration (s)			
	Trial 1	Trial 2	Trial 3	Mean ± SD
L	**115**	120	120	118.3 ± 2.9
M	**120**	120	120	120.0 ± 0.0
N	**82**	60	32	58.0 ± 25.1
O	49	0	0	16.3 ± 28.3
P	22	9	16	15.7 ± 6.5
Q	9	0	19	9.3 ± 9.5
R	0	0	0	0.0 ± 0.0
S	34	120	120	91.3 ± 49.6
T	11	73	10	31.3 ± 36.1